This study evaluates the polyphenolic and volatile profile of wines and by-products from Macedonia produced under different vinification procedures. The first goal of this study was the isolation of anthocyanins from grape pomace of three red varieties (Vranec, Merlot and Pinot Noir) by application of countercurrent chromatography. The major anthocyanin was malvidin-3-glucoside and also delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-p-coumaroyl-glucoside were isolated. The "color activity concept" was applied and visual detection thresholds of isolated anthocyanins were determined. The content of trans-resveratrol and its glucoside piceid, resulting antioxidant activity and the relationship between their levels and winemaking technology has been studied in Macedonian red wines from the two main grape varieties Vranec and Merlot. Finally, the liberation of glycosidically bound volatile compounds from white and red wines was performed by using the enzyme mixture "AR 2000" and the obtained volatile profile was compared with that of the wines in which the enzyme "Ednozym Aromatic" during vinification was applied.

Sanja Kostadinovik Velickovska

Dr. Sanja Kostadinović Veličkovska was born 03.28.1979 in Macedonia. She was awarded a DAAD research grant for PhD. She has obtained her PhD degree in Food Chemistry at the Institute of Food Chemistry, Technical University of Braunschweig, 2012. Currently, she is working at the Faculty of Agriculture, University "Goce Delčev" in Štip, Macedonia.

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S. Kostadinovik, M. Stefova, H. Mirhosseini, S. Tolle, G. Milanov, V. Ivanova and P. Winterhalter, Direct immersion solid phase microextraction-gas chromatography/mass spectrometry for isolation of flavor compounds from Temjanika/Muscat white wines

12th International Flavor Conference, 4th George Charalambous Memorial Symposium, Island of Skiathos, Greece, 2009

S. Kostadinovik, B. Vojnoski A. Wilkens, M. Stefova, V. Ivanova and P.Winterhalter, Occurance of resveratrol in Vranec and Merlot wines from Macedonia: effect of variety and enological practices

XXI Congress of Chemists and Technologists of Macedonia, Ohrid, Macedonia, 2010

To my parents and my sister Dragana

With love to my husband Igor

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PREFACE

Wine and grape pomaces contain complex mixtures of polyphenolic compounds belonging to flavonoids and non-flavonoids which are considered to be responsible for the health impact in human nutrition. Additional compounds are volatile constituents such as aldehydes, esters, alcohols, acids, which are responsible for the flavor.

This PhD thesis evaluates the polyphenolic and volatile profile of commercial red and white wines as well as the red varieties (Vranec and Merlot) from Macedonia produced under different vinification procedures.

A further goal of this PhD thesis was the isolation of anthocyanins from grape pomace of three red varieties (Vranec, Merlot and Pinot Noir) by application of countercurrent chromatography. After purification of the fractions by means of preparative high performance liquid chromatography, the structures of isolated pigments were elucidated by electrospray ionization multiple mass spectrometry and nuclear magnetic resonance (NMR) spectroscopy. The major anthocyanin was malvidin-3-glucoside and also delphinidin-3-glucoside, cyanidin-3-glucoside, petunidin-3-glucoside, peonidin-3-glucoside and malvidin-3-p-coumaroyl-glucoside were isolated.

The “color activity concept” was applied for the first time to the anthocyanin mixtures obtained from grape pomace from Macedonian wines and visual detection thresholds of isolated anthocyanins were determined. The results of the “color activity value” of isolated pigments and their detection thresholds were in good agreement with the color of the different varieties of red grape pomace.

The content of *trans*-resveratrol and its glucoside piceid as major stilbenes in wines and the relationship between their content and winemaking technology has been studied in Macedonian red wines from the two main grape varieties Vranec and Merlot. This part of PhD thesis has been carried out to give an insight into the concentration of stilbenes in the wines prepared from the regionally specific Vranec variety as well as from the international variety Merlot. Moreover, the effect of winemaking technology including time of maceration, type of yeast and the level of sulfur dioxide applied on the stilbenes content has been studied. The results imply that the most important factor in winemaking technology affecting the stilbenes content is the maceration time since the highest concentrations of *trans*-resveratrol and piceid were found in red wines from both varieties produced with 6 and 10 days of maceration. Concerning the yeast type, higher concentration of *trans*-resveratrol and piceid have been obtained with French yeast in comparison to Macedonian yeast. Higher concentration of SO_2 protects the phenolic compounds in wines from oxidation during the winemaking process, but it does not affect significantly the concentration of both analyzed stilbenes in wines. The obtained results contribute to the understanding of the process of winemaking in terms of the stilbenes content.

Wines are known to contain complex mixtures of polyphenolic compounds. These compounds have antioxidant activity and are likely to protect the organism against cardiovascular and other degenerative diseases. The antioxidant activity depends on the type of polyphenolic compounds. A common method for determination of the antioxidant activity of wines is the 2,2-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) method. This method was used for testing twelve Vranec and

twelve Merlot wines obtained under different vinification conditions (different maceration times, different yeasts for fermentation, and two levels of SO_2). Hence, the next objective of this PhD study was to investigate the influence of wine-making technology on antioxidant activity and sensory profile of the wines mentioned above. More precisely, the effect of processing conditions namely type of yeast, maceration time and concentration of SO_2 on antioxidant activity and sensory profile of 24 Macedonian red wines were studied. The results indicated a significant effect of the maceration time on antioxidant activity of wines with the lowest antioxidant activity measured for the wines produced with 3 days of maceration and highest values for the wines obtained with 6 days for Vranec and 10 days of maceration for Merlot wines. The antioxidant activity is slightly higher by application of Macedonian yeast compared to French yeast. Also higher concentrations of SO_2 in Vranec wines resulted in a higher antioxidant capacity.

Finally, the volatile profile of commercial white and red wines from Macedonia was subject of this PhD thesis. The smell and the taste of the wines are due to a complex mixture of many volatile compounds. These compounds have distinct physicochemical and sensory properties regarding, for example, polarity, volatility, and odour impact. In this study, a head space solid-phase microextraction (HS-SPME) procedure was optimized and applied for sample preparation of 15 of the most famous commercial wines from Macedonia followed by separation and detection with GC-MS. Separation of the compounds was performed on a Carbowax column after the injection of the CRB-DVB-PDMS fiber in splitless mode. Liberation of glycosidically bound volatile compounds from white and red wines was performed by using the enzyme mixture “AR 2000” and the

obtained volatile profile was compared with that of the wines in which the enzyme “Ednozym Aromatic” during vinification was applied.

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1. INTRODUCTION

The primary meaning of the word “wine” is the product of the fermentation by yeasts of the sugars in the juice of grapes resulting in an alcohol content of 9-15 % v/v (per cent by volume) (Clarke & Bakker, 2004). Table wines can be red, rosé and white, the colors depending on the choice of grapes and the wine-making processes used. Although red wines tend to be dry, white wines are produced from dry to very sweet (Ribéreau-Gayon & Glories, 2006).

There is much historical information on wine dating since Stone Age, some 6000 years ago from Mesopotamia and Egypt, as a symbol of Jesus blood in Christianity and as one of the most popular alcoholic drinks today over the world.

In terms of chemistry, wine is the complex mixture of a large number of compounds including polyphenolic compounds such as anthocyanins and stilbenes responsible for likely health effects and esters, alcohols, aldehydes, acids responsible for the smell and the taste of the wine.

Polyphenolic and volatile compounds are the major contributors to the color and flavor of wine. These components are mainly presented in the skins and seeds of the grape and are extracted during the wine-making process. Polyphenols play a double role during vinification. They determine the overall quality (color and taste) of red wines and also exhibit bactericide, and antioxidant properties that apparently protect consumers from cardiovascular disease (Ribéreau-Gayon & Glories, 2006).

Anthocyanins are the most abundant polyphenols responsible for the color of red grapes. The study of anthocyanins as natural colorants is an extensive area of investigation due to the growing interest of substituting

synthetic colorants by natural ones. Anthocyanins (of the Greek anthos = flower and kianos = blue) are the most important water-soluble pigments of the plants. These pigments are responsible of the shiny orange, pink, red, violet and blue colors in the flowers and fruits of many plants. Anthocyanins exhibit a range of biological activities. The contribution of these substances to health-promoting effects is under current investigation. The most important property of anthocyanins is their antioxidant activity, which plays a main role in the prevention of neuronal and cardiovascular illnesses, cancer and diabetes (Moreno-Arribas & Carmen Polo (eds.), 2009; Jackson, 2008; Bakker & Timberlake, 1997; Castañeda-Ovando *et al*., 2009). The structure of anthocyanins and their properties as colorants in plants were subject of investigations of Brouillard *et al*. (2003). Radical scavenger activity of anthocyanins and their aglycons were also studied in the work of Kähkönen & Heinonen (2003).

Antioxidant activity of anthocyanins and their role as natural colorants were the main reasons for their identification, quantification and isolation from a wide range of different plants. Grape skin contains a great number of anthocyanins, the concentration of which not only varies considerably according to the grapevine variety but also due to climatic and environmental factors.

Anthocyanin compounds are mainly located in the grape skins, with the exception of the Teinturier varieties that also contain anthocyanins in the pulp. The anthocyanins identified in grape skins and wines from *Vitis vinifera* are the 3-*O*-monoglucosides and the 3-*O*-acylated monoglucosides of five main anthocyanidins – delphinidin, cyanidin, petunidin, peonidin and malvidin – which differ from each other by the number and position of the hydroxyl and methoxyl groups located in the aromatic rings of the

molecule (Fig 1-5). Acylation occurs at the C-6 position of the glucose molecule by esterification with acetic, *p*-coumaric and caffeic acids. Recently, the existence of anthocyanins acylated with lactic acid has been reported (Ribéreau-Gayon & Glories, 2006; Moreno-Arribas & Carmen Polo (eds.), 2009; Jackson, 2008; Bakker & Timberlake, 1997; Castañeda-Ovando *et al.*, 2009; Brouillard *et al*., 2003; Kähkönen & Heinonen, 2003). Accumulation of anthocyanins starts at veraison, i.e. the period of berry ripening, when changes of colors occur (green color is changing to yellow-green for white grapes or red and different blue nuances for red grapes).

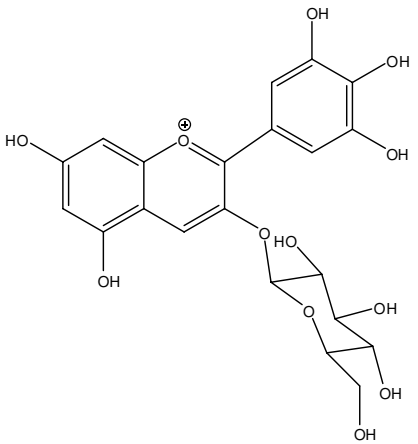


Fig. 1. Structure of delphinidin-3-*O*-β-D- glucopyranoside (delphinidin-3-glucoside)

1. Introduction

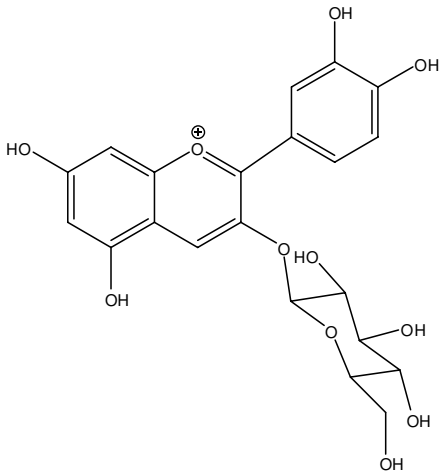


Fig. 2. Structure of cyanidin-3-*O*-β-D-glucopyranoside (cyanidin-3-glucoside)

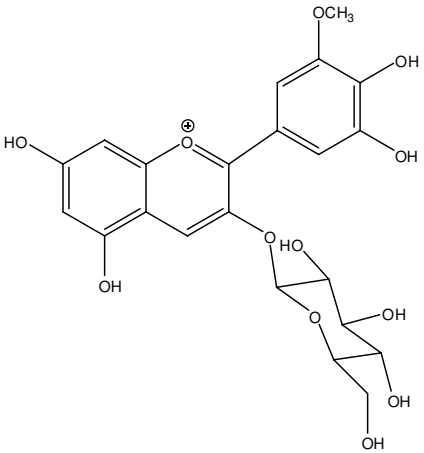


Fig. 3. Structure of petunidin-3-*O*-β-glucopyranoside (petunidin-3-glucoside)

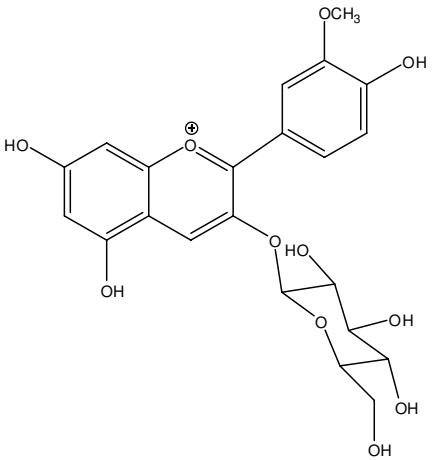


Fig. 4. Structure of peonidin-3-*O*-β- glucopyranoside (peonidin-3-glucoside)

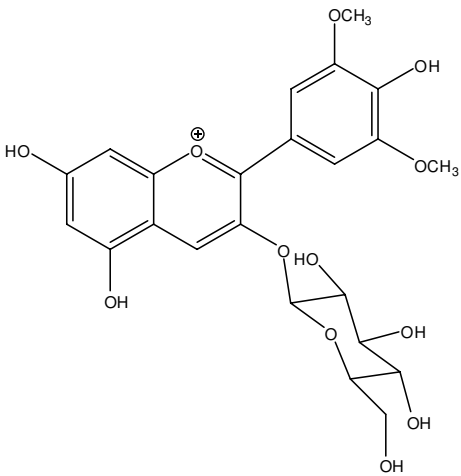


Fig. 5. Structure of malvidin-3-*O*-β-glucopyranoside (malvidin-3-glucoside)

High-performance liquid chromatography (HPLC) is the most widely used technique for identification and quantification of anthocyanins on an analytical scale and countercurrent chromatography for isolation of

anthocyanins from grape skin on a preparative scale, respectively (Cantos *et al*., 2002; Luque-Rodríguez *et al*., 2007; Mateus *et al*., 2002; Revilla *et al*., 2001; Valls *et al*., 2009; Winterhalter, 2007; Degenhardt *et al*., 2000b; Gutzeit *et al*., 2007). In the work of Schwarz *et al*. (2003) several hundred milligrams of anthocyanins were isolated during a single run using large-scale countercurrent chromatography.

Grape pomace consists of the skin, stems, and seeds of grapes that remain after processing in the wine and juice industry (Chinnici *et al*., 2009). A chemical study of the constituents of grape pomace from a *Vitis Vinifera* cultivar largely employed in Sicily for red wine production, namely '*Nerello Mascalese*', was reported (Amico *et al*., 2004). Evaluation of the radical scavenging activity and polyphenol content of pomace samples obtained from different wineries or with different procedures from Sicily were also subject of study (Amico *et al*., 2008). Kammerer *et al*. (2004) screened polyphenolic compounds from different types of red and white grape pomaces. In the work of Monrad *et al*. (2010) supercritical solvent extraction was applied as powerful extraction technique for isolation of anthocyanins from red grape pomace. Isolation of the anthocyanins from wine grapes with step gradient and from different wine fractions was also studied (Vidal *et al*., 2004; Salas *et al*., 2005). Structure elucidation of isolated anthocyanins using NMR spectroscopy confirmed the structure of the most abundant pigments in plants (Ha *et al*., 2010; Wolniak & Wawer, 2008; Nickavar & Amin, 2004; Tsuda *et al*., 1994; Takeoka *et al*., 1997; Kuskoski *et al*., 2003; Jordheim et al., 2007; Andersen *et al*., 1995; Atanasova *et al*., 2002; Gueffroy *et al*., 1971, Oliveira *et al*., 2006; Aguirre *et al*., 2010; Yawadio *et al*., 2007). The contribution of particular

anthocyanins to the overall color of red wines was studied by Degenhardt *et al.* (2000a).

Due to the great importance of anthocyanins as antioxidants as well as natural pigments for coloring of food and the fact that large quantities of grape pomace are discarded every year during wine production in Macedonia, part of this PhD work was directed to explore possibility in using grape pomace as low cost raw material for the isolation of high valuable pigments with functional properties.

In the present study the isolation of anthocyanins from the Macedonian red grape pomace from the grape varieties "Vranec", "Merlot", and "Pinot Noir" by application of chromatographic methods, in particular high-speed countercurrent chromatography, will be presented.

„Vranec"

"Vranec" is the most widely cultivated and the most important variety in the Republic of Macedonia being used for production of high quality red wines. Outside the Republic of Macedonia, it is primarly planted in Montenegro (autochthonous variety), Serbia and Croatia (Dalmatia). Within Macedonia, it is planted in the wine region of Povardarie, the most famous region for wine growing and winemaking, where more than 80 % of the Macedonian vineyards are located. The "Vranec" grape is depicted in Fig. 6. The berry is medium sized with elongated shape. The skin is dark-blue colored, covered with bloom. With regard to susceptibility to diseases, "Vranec" is sensitive to downy mildew (*Plasmopara viticola*) and more resistant to powdery mildew (*Uncinula necator*) and grey mold (*Botrytis cinerea*). In addition, this grape variety is sensitive to low winter temperatures. It is high yielding, producing fruit that is deeply colored.

The wines produced from this variety have intense dark red, ruby color and strong aroma of mixed dried plums, blackberries and currants (Bozinović, 1996).

Fig. 6. Vranec grape variety (Wines of Macedonia, 2011)

„**Merlot**“

“Merlot” is widely cultivated throughout the world, and it is one of the primary grapes in the Bordeaux wine region. It is also cultivated in the Republic of Macedonia, especially in Tikveš, Skopje, Ovče Pole, Ohrid and Kumanovo vineyard areas. The cluster of “Merlot” is medium sized, with pyramidal and pyramidal-round shape (Fig. 7). The berry is small and round, with thick and firm skin, covered with bloom. This variety gives the highest yields at fertile and temperate moist soils and it is middle resistant to drought. With regard to disease susceptibility, “Merlot” is high resistant to powdery mildew and grey mold, and sensitive to downy mildew. This grape variety shows middle resistance to low winter temperatures. Merlot wines have ruby-red color and aroma of ripe grapes, honey and mixed caramelized forest fruits (Bozinović, 1996).

Fig. 7. Merlot grape variety (Wikipedia “Merlot”)

„**Pinot Noir**“

The name “Pinot Noir” is derived from the French words for "pine" and "black" alluding to the grape variety's tightly clustered dark purple pine cone-shaped bunches of fruit (Fig. 8).

Some viticultural historians believe this shape may have given rise to the name. Pinot noir tends to produce narrow trunks and branches. In the vineyard it is sensitive to light exposure, cropping levels, soil types and pruning techniques. In the winery it is sensitive to fermentation methods, yeast strains and is highly reflective of its *terroir* with different regions producing very different wines. Its thin skin makes it highly susceptible to bunch rot and other fungal diseases. The vines themselves are prone to downy mildew, leaf roll, and fanleaf. These complications have given the grape the reputation of being difficult to grow. Ancient Romans knew this grape as Helvenacia Minor and vinified it as early as the first century.

Recognized worldwide as a great wine grape, Pinot Noir has many alias and is grown in Algeria, Argentina, Australia, Austria (called Blauburgunder or Spätburgunder), Brazil, Canada, Croatia (Burgundac), Czechoslovakia, England, France, Germany (Spätburgunder), Greece, Hungary, Italy (Pinot Nero), Mexico, New Zealand, Switzerland (Clevner, but labeled "Dole" when often blended with Gamay Noir), and the United States. It is also cultivated in the Republic of Macedonia (Tikveš wine-growing region). The wines from "Pinot Noir" grape variety have a deep red color and taste of ripe fruit with strong woody notes.

Fig. 8. Pinot Noir grape variety (Wikipedia “Pinot Noir”)

The structures of the isolated and purified anthocyanins from the three grape varieties mentioned above were elucidated by NMR spectroscopy. NMR data confirmed the most abundant anthocyanins responsible for the color of the grape pomace. Also, the “color activity concept” was performed for investigation of the particular contribution of the isolated pigments to the color of the three different varieties of grape pomace.

Apart from anthocyanins, the next class of polyphenols responsible for the “French Paradox” is the class of stilbenes. Originally epidemiological studies indicated an inverse relationship between moderate wine consumption and risk of coronary heart disease, the so-called ‘‘French

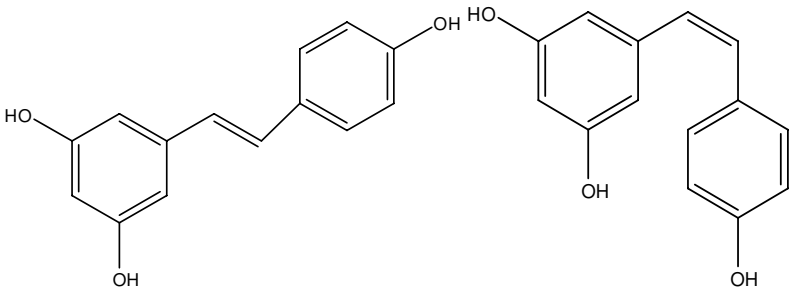
Paradox''. The "French paradox" refers to the comparatively low incidence of heart disease among the French population, in spite of a considerable fat intake (Renaud & de Lorgeril, 1992).

Stilbenes belong to the non-flavonoid class of wine phenolic compounds, and resveratrol is the major stilbene present in grapes and wines (Rentzsch *et al*., 2008). Resveratrol occurs in two isomeric forms, the *trans*- and *cis*-configured isomers. *Trans*-resveratrol or *trans*-3,5,4'-hydroxystilbene is the most abundant form and is mainly located in grape skins (Bravo *et al*., 2008). *Cis*- resveratrol can be obtained from the *trans*- form by isomerization using heat or UV radiation. Glycosidic conjugate forms of *trans*- and *cis*-resveratrol are known as piceids (Fig. 9).

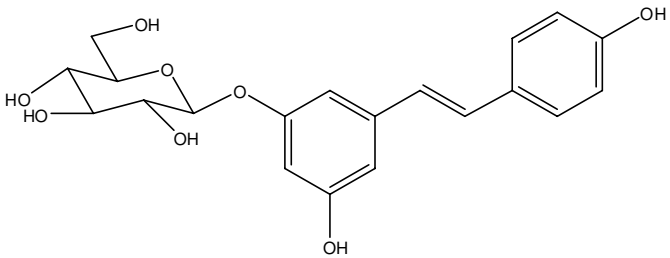
Generally, stilbenes are known as phytoalexins which can be biosynthesized from grapevines as a defence to fungal diseases, such as *Botrytis cinerea* or abiotic stress, such as UV irradiation.

In a study of Wenzel *et al*. (2005) the implications of selected chemopreventive parameters and metabolic conversion of resveratrol in vivo were investigated. In order to reveal information on absorption, metabolism, and the consequent bioavailability of resveratrol, different research approaches were performed, including in vitro, ex vivo, and in vivo models, all of which were considered. According to the findings of the working group of Samoza, the oral bioavailability of resveratrol was almost zero due to rapid and extensive metabolism and the consequent formation of various metabolites as resveratrol glucuronides and resveratrol sulfates (Wenzel & Samoza, 2005).

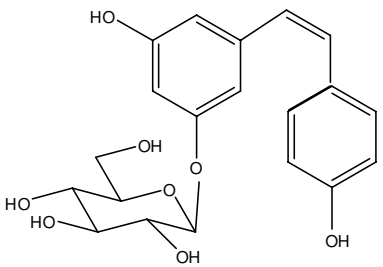
The antioxidant and antimicrobial activity of resveratrol is likely to provide health benefits, such as the prevention of cardiovascular diseases, arteriosclerosis, and cancer.



trans-3,4',5 –trihydroxystilbene (trans-resveratrol) *cis*-3,4',5 -trihydroxystilbene *(cis*-resveratrol)



trans-resveratrol-3-O-β-glucopyranoside (*trans*-piceid)



cis-resveratrol-3-O-β-glucopyranoside (*cis*-piceid)

Fig. 9. Structure of resveratrol derivatives

Thus, the interest of the scientific community in the phytoalexin resveratrol has increased over the last years (Barlass *et al*., 1987; Creasy & Coffee, 1988; Dercks & Creasy, 1989). It was reported that the application of enzymes (glycosidases) increases the concentration of resveratrol. In the study of Todaro *et al*. (2008), purified *β*-glucosidase from *A. Niger* was able to raise *trans*-resveratrol levels without significant alteration of physicochemical properties of red wines in comparison with commercial *S. cerevisiae* yeast strains (Frankel *et al*., 1993). Thus, relationships between the stilbene composition in grape skins and that in the corresponding wines were studied. According to findings of Sun *et al*. (2006), the total amount of piceid might predict the level of stilbenoids in the respective red wines. Comparison between levels of resveratrol in different red wines from a single grape variety (mono-varietal red wines), and the level of resveratrol in red wines from different regions was also established with conclusion that the average level of *trans*-piceid is as much as three times higher than that of *trans*- resveratrol. The content of resveratrol was determined in many wines, e.g. from Greece white (0.005-0.57 mg/L) and red wines (0.550-2.534 mg/L) (Gerogiannaki-Christopoulou *et al*., 2006; Kallithraka *et al*., 2001), red wines from Canary Island (2.06-3.75 mg/L) (Rodriguez-Delgado *et al*., 2002), wines produced from grapes cultivated in the Snake River Valley of Idaho (> 1.91 mg/L) (Lee & Rennaker, 2007), red and rosé wines produced in the four designations of origin of Aragon (0.62-3.09 mg/L) (Abril *et al*., 2005), red wines elaborated from Galician varieties (3.02-36.13 mg/L) (Feijóo *et al*., 2008), Brazilian wines (0.04-1.26 mg/L) (Lucena *et al*., 2010) as well as in a wide range of commercial red and white wines from Japan (0.2-1.5 mg/L) and France (3.8-7.4 mg/L) (Stervbo *et al*., 2007). The concentration of *trans*-resveratrol in wines from the

Hungarian Villàny region was in the range from 0.8-3.9 mg/L (Pour Nikfardjam *et al*., 2006).

There are several papers which examined the impact of technological practices on the levels of resveratrol. Effects of some prefermentative operations (e.g. addition of SO_2 and ascorbic acid before grape crushing or must hyperoxidation) on the final resveratrol content in red wine have been studied. The addition of charcoal and PVPP eliminated *trans*- and *cis*-resveratrol in wines (Castellari *et al*., 1998). The relationship between free and bound forms of resveratrol are discussed, taking into account the role of some main factors, such as extraction from grape skin, hydrolysis of glucosides, and *trans*- *cis* isomerization. The results obtained from the study showed complete extraction of *trans*-resveratrol between 6 and 10 days of maceration time (Soleas *et al*., 1995). Different maceration methods for the extraction of stilbene compounds were evaluated as well as the influence of the activity of wine microorganisms. It has been demonstrated that lactic acid bacteria can impact both resveratrol and piceid levels in wines (Poussier *et al*., 2003). Increasing resveratrol levels in Cynthiana and Nobles wines by using ultra violet light, effect of variety and the effect on carbon dioxide on resveratrol concentration in wines have been studied with conclusion that exposure of ultraviolet light significantly increases the resveratrol level (Threlfall *et al*., 1999). Infection of grapes with *Botrytis cinerea* also affects the level of resveratrol in wines. In the study of Jeandet *et al*. (1995) higher infection of *B. cinerea* decreases the concentration of resveratol in wines most likely because of degradation effects of enzymes of this fungus. According to the findings of Vrhovsek *et al.* (1997), different yeast strains can significantly affect the resveratrol content in wine, except that of the *cis*-resveratrol glucoside. Increase of the

level of resveratrol after malolactic fermentation resulted from glycoside cleavage of piceid primarily concentrated in the skin of the grapes (Pezet & Cuenat, 1996). A decrease in the concentration of piceid and a simultaneous increase of the free aglycon during maceration was reported (Mattivi *et al*., 1995). Influence of thermovinification and cold soaking on the level of *trans*-resveratrol and piceid has been evaluated (Clare *et al*., 2004). Further studies have examined the effect of different types of yeast (Vacca *et al*., 1997) and the improvement of enzymatic hydrolysis with regard of higher levels of resveratrol in wine (La Torre *et al*., 2004). Evolution of resveratrol and piceid contents during the industrial winemaking process of sherry wine showed that the flor yeast and biological aging stage are responsible for the decreased level of resveratrol (Roldan *et al*., 2010). Influence of UV treatment of the level of stilbenes in the wine was examined by Guerrero *et al.* (2010). Grapes of the UV batch showed increased content in piceatannol, *trans*-resveratrol and viniferins. Although *trans*-resveratrol concentration decreased progressively during winemaking, especially during alcoholic fermentation, a few times higher concentration of *trans*-resveratrol in wine produced from UV treated grapes was noted (Guerrero *et al*., 2010).

Referring to the discussion above, there are already many published studies performed on resveratrol in wine and grape, but, till now, there are no published results about the level of resveratrol in Macedonian red wines. Therefore, the second objective of this PhD work was to examine the content of resveratrol and its *ß*-D-glucosides (piceids) in Vranec and Merlot wines produced under different winemaking conditions. This includes different maceration times (3, 6 and 10 days), SO_2 content and different types of yeast. The relationship between varieties and resveratrol

content will be examined as well as the effect of maceration time, effect of yeast and impact of different concentration of SO_2 as an antioxidant on resveratrol content in the wines. High-performance liquid chromatography coupled to mass spectrometry and diode array detection will be applied for identification and quantification of stilbenes after direct injection of the wines.

Both classes of polyphenolic compounds (anthocyanins and stilbenes) discussed above are known to possess antioxidant properties and the antioxidant potential of wine is largely attributable to these polyphenols (Zenebe & Pechanova, 2002). Among the numerous polyphenolic wine constituents especially those compounds with an ortho-diphenolic partial structure have been determined as efficient radical scavengers (Rice-Evans *et al*., 1996).

A common method for determination of the antioxidant activity of wines is the 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) radical cation ($ABTS^{\bullet+}$) method (Villaño *et al*., 2004). The TEAC (Trolox Equivalent Antioxidant Capacity) value which is measured for wine samples, expresses the concentration of a Trolox solution (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid) whose antioxidant activity is identical to that of the wine itself. This index is defined as the millimolar concentration of a Trolox solution whose antioxidant capacity is equivalent to a 1.0 mM solution of the sample under study. Villaño *et al.* (2004) have used different dilutions and reaction times for measuring absorbance and the optimal conditions depending on the characteristics of the wine tested (white, red and sherry wines). They found that TEAC values are time-dependent and demonstrated the increase of the values in wines by 30–40% between $TEAC_2$ min. and $TEAC_{15}$ min. Results obtained from

measurements of the wine samples showed 10 times higher values from red wines in comparison of the results obtained from the white wines due to higher concentration of phenolic compounds. Difference between white and sherry wine was not statistically significant (Villaño *et al*., 2004). Several papers have been published with regard to correlations of antioxidant activity of wines and polyphenolic composition. In the work of Fernández-Pachón *et al*. (2004) 50 % of the total red wines scavenging radical activity was attributed to polymeric phenolic compounds and the rest was in favor of anthocyanins and flavan-3-ols and less in favor of phenolic acid and flavonols. Paixão *et al.* (2007) explained high correlation between total phenolic content and antioxidant activity and confirmed highest antioxidant activity for red wines. Villaño *et al.* (2005) found that phenolic metabolites have a non-negligible antioxidant activity and their values are similar to those of phenolic compounds themselves. Staško *et al*.(2008) investigated 86 red and white wines from the Slovak and Austrian and found approximately 10 times greater antioxidant activity of red wines in comparison to white wines.

Also, there are published results for the contribution of the anthocyanin fraction to the antioxidant properties of wine. According to the findings of Rivero-Pèrez *et al.* (2008a) isolated anthocyanins from wines exhibited important antioxidant properties and indicate to be some of the most important components responsible of the antioxidant capacities of the red wines. Anthocyanins show a high protective effect as scavengers (hydroxyl and superoxide radicals) and a significant capacity to transfer electrons. Rivero-Pèrez *et al.* (2008b) studied the influence of aging on the antioxidant potential of wine. The results obtained in the study indicated that the antioxidant potential of wines was not influenced by

microoxigenation. Recently, results were published trying to explain the relationship between antioxidant activity of wine and the applied wine-making process. Lachman *et al.* (2007) investigated the total antioxidant status of two white and two red wine varieties from the Žernoseky wine region and detected that the red wine variety shows much higher level of total antioxidant status than white wines. Baiano *et al.* (2009) found that wine obtained through traditional technology and addition of tannins showed the highest antioxidant activities (according to the DPPH-assay) and wines produced through traditional technology were the richest in anthocyanins.

Examination of antioxidant activity of aged red wines showed that total flavanols are the class of polyphenols that account for hydroxyl free radical scavenging activity and to a lesser extent for antiradical and reducing ability. The relationship between the antioxidant properties and the total phenolics was less significant and finally in aged wines very weak correlation was found with the total anthocyanin content (Arnous *et al*., 2001). Main polyphenolics identified in the selected aged Spanish red wines such tannic and gallic acid were identified as most responsible for radical scavenging capacity (Larrauri *et al*., 1999). Examination of eight Italian young wines *Vini Novella* prepared using carbonic maceration showed that antioxidant potential of the wines is much dependent on aging and less dependent on the wine-making technology applied (Pellegrini *et al*., 2000). Results obtained from some red and white wines from Greece indicated that wine aging may affect their antioxidant activities, while anthocyanins and flavanols may be among the most active phenolics (Roussis *et al*., 2008). The relationship between concentration of stilbene and resulting antioxidant activity of wines from the region of Idaho was

also object of study (Lee & Rennaker, 2007). Correlation between antioxidant activity and polyphenols separated by HPLC in Mencía and Brancellao wines indicated significant activity of polymerized polyphenol fractions (Alèn-Ruiz *et al*., 2009). Ginjom *et al*. (2010) found positive correlation between phenolic compounds and antioxidant activity of Australian red wines. According to their results, the most abundant polyphenols were tannins, while monomeric anthocyanins were responsible for less than 10 % of the activity. The study of 24 red, 11 white and 2 rosė wines obtained from six provinces in China indicated that the results of the ORAC, DPPH and ABTS methods are in relatively good agreement which confirmed that any of these methods can be used for the evaluation of the antioxidant capacity of wines (Li *et al*., 2009).

Influence of temperature, pre- and postfermentative factors, influence of yeasts and other factors during wine-making process on the phenolic compounds of wines was object of many studies (Gil-Muñoz *et al*., 1999; Gil-Muñoz *et al*., 1997; Caridi *et al*., 2004; Mazza *et al*., 1999). Villaño *et al*. (2006) studied the influence of enological practices on the antioxidant activity of wines. They confirmed that maceration time and variety of grapes had impact on the antioxidant activity of the wines and clarification treatments did not affect significantly the level of phenolic compounds. Influence of vinification on the antioxidant activity of the wines was object of study in the work of Burns *et al*. (2001).They concluded that 9 days of maceration are sufficient for complete extraction of polyphenolic compounds because, in accordance to the results obtained, wines reached maximal value for antioxidant activity during the storage as resulting of condensation products (Burns *et al*., 2001). Spranger *et al*. (2004) found differentiation between wines produced by skin and carbonic maceration. It

was concluded that wines produced by application of skin maceration were richer in polyphenols compared to wines produced by carbonic maceration, but during increased maceration time the level of total and some individual anthocyanins decreased. Kovac *et al*. (1992) found that the length of maceration time and addition of high quantities of pomace, seeds and must increased the concentration of catechines and proanthocyanidins in wines. Budić-Leto *et al*. (2008) studied the influence of maceration time on the concentration of polyphenolic compounds in autochthonous cultivar "Plavac mali" (*Vitis vinifera* L.). The results obtained from examinations indicated that prolonged maceration can increase the concentrations of proanthocyanidins and decrease the content of anthocyanins. The effect of SO_2 on polyphenolic compounds in model wine was object of the investigation of Danilewicz *et al*. 2008) who clearly showed that SO_2 did not react directly with oxygen to protect polyphenols in wine, because of its autoxidation. In the work of Tao *et al*. (2007) it was shown that concentration of SO_2 is in direct correlation with degree of polymerization of wines. Results showed that wines with higher concentrations of SO_2 inhibited the possibility of polymerization and formation of polymeric pigment and changes in the structure of tannins.

The third part of this PhD work was the evaluation of antioxidant activity of the most popular red wines in Macedonia from Vranec and Merlot grape varieties.

To the best of our knowledge there are no published results for antioxidant activity of wines from the region of Macedonia. So, the main objective of the present study was to measure the antioxidant activity of Vranec and Merlot wines produced under different wine-making techniques or more precisely to understand the effect of processing conditions i.e., type of

yeast, maceration time and content of SO_2. The ABTS method (TEAC assay) was used for the analysis of twelve Vranec and twelve Merlot wines. Statistical evaluation was applied in order to find relationships between antioxidant activity of the wines and the applied techniques for wine-making. The effect of wine-making on sensory profile of wines was also estimated.

Beside the polyphenolic profile, different vinification procedures determine also the volatile profile responsible for the aroma and the taste of the wine. Many papers established relationships between volatile profile of wines and their wine-making procedure. The influence of commercial and locally identified yeasts on the quality parameters of young Cabernet Souvignon wines was studied (Sharma *et al*., 2009). The effect of yeast on the overall organoleptic taste of wines was studied with conclusion that different yeast stains affect significantly the volatile compounds and hence the overall organoleptic taste of wines (Torrens *et al*., 2008). In the study of Domizio *et al*. (2007) the influence of yeasts on the organoleptic taste of Vinsanto wines was estimated. The work of Callejon *et al*. (2010) proved that different autochthonous yeast strains were responsible for different volatile profiles of red wines. In the work of Kotseridis *et al*. (2000) Merlot and Cabernet sauvignon wines had very similar aroma and the most significant differentiation between the wines was the level of 4-hydroxy-2,5-dimethylfuran-3(2H)-one (HDMF). Sánchez-Palomo *et al*. (2007) confirmed that different wine-making procedures affect the sensory profile of wines. Skin-contact treatment enhanced the sensory attributes as sweet, peach, apricot and green apple note and also influenced the intensity, body and fruitiness of the wines.

1. Introduction

Volatile compounds of white and red wines have distinct physicochemical and sensory properties regarding, for example, polarity, volatility, and odour impact as a result of the functional groups (alcohol, aldehyde, acid, etc.) being present in their molecules. They have three different origins: (i) from the grape (pre-fermentative aroma); (ii) from the yeast during the first or second fermentation (fermentative aroma); and (iii) from the aging process (post fermentative aroma) (Sarrazin *et al*., 2007). Wine volatiles have an exceptional range of aromas, from citrus and dried fruit in young wines, orange peel in older wines, and honey or waxy nuances in wines subjected to oxidative aging (Sarrazin *et al*., 2007).

Solid-phase microextraction (SPME) is a suitable technique for the extraction of volatile compounds from the complex wine matrix. In the work of Tat *et al*. (2005) CRB-DVB-PDMS fiber was reported to have the best performace for extraction of aromatic fractions in wines. Vaz Freire *et al*. (2001) found PDMS coating as most suitable for the analysis of esters and polyacrylate coating as most suitable for alcohols and terpines in wines. 32 wine esters in 19 French wines were identified and quantified using HS-SPME even in trace quantities (ng/L) (Antalick *et al*., 2010). Volatile profile of sparkling wines determined by three extraction techniques showed that SPME technique is sensitive enough and comparable with solid phase extraction (SPE) and liquid-liquid extraction (Bosch-Fuster *et al*., 2007). Effect of sample matrix on the volatile compounds in wines was also studied. In the work of Whiton *et al*. (2000) influence of alcohol matrix on extraction of volatile compounds by using HS-SPME was explained. Validation of a HS-SPME method for of the identification and quantification of wine volatiles using several internal standards was reported in the work of Howard *et al*. (2005). According to

their results the most appropriate fiber for extraction of wine volatiles was CRB-DVB-PDMS fiber in combination with GC-MS. In the work of Galvan *et al*. (2008) CAR/PDMS fiber was described as most appropriate for extraction of 3-alkyl-2-methoxypyrazines in Frontenac and Leon Millot wines. Also, quantification of volatile compounds of 27 Greek wines was obtained by using HS-SPME with 85 μm polyacrylate coating fiber (Koussissi *et al*., 2007). High concentrations of volatile compounds as terpenes were also found in grapes and wines in bound form as non-volatile glycosides. Isolation of glycosidically bound compounds from Riesling leaves and wines has been performed by Winterhalter (1991) and Winterhalter *et al*. (1991) Noble *et al*. (1988) found that the cleavage of glycosides of terpenes increase the bitter taste of Muscat of Alexandria wine. *β*-Glucosidase, *α*-arabinosidase and *α*-rhamnosidase activities of three enological yeast strains during winemaking did not show significant difference in the sensory analysis of the produced wines (Delcroix *et al*., 1994). Also, the stability of free and glycosidically-bound fractions of some components of Muscat grape aroma (terpenols and aromatic alcohols) was investigated during alcoholic fermentation and in wines of different ages. The obtained results showed that concentration of free linalool and α-terpineol increased while geraniol and nerol decreased in older wines (Gunata *et al*., 1986). The distribution of free and glycosidically-bound monoterpenes among skin, juice, and pulp fractions in Muscat of Alexandria, white Frontignac, and Traminer grapes was evaluated (Wilson *et al*., 1986). In the work of Tate *et al*. (1986) wines produced with *S. bayanus* strain EC1118 and *S. cerevisiae* strain VL1 increased melon and muscat aroma. Effect of carbonic anaerobiosis on the changes of concentration of free and bound volatile compounds after nine days in

Muscat de Frontignane wines was studied by Bitteur *et al*. (1992). Isolation of glycosidically bound volatile compounds from white and red wines and effect of "AR 2000" enzyme on the glucose bound volatile compounds were studied by several authors (Sánchez Palomo *et al*., 2006; Sánchez Palomo *et al*., 2007; Ugliano & Moio, 2006; Vilanova *et al*., 2007; Castro Vázquez *et al*., 2002; Gómez García-Carpintero *et al*., 2011; Kang *et al*., 2010).

The last goal of this PhD work is to investigate the volatile profile of fifteen of the most famous white and red wines from Macedonia. Solid-phase microextraction coupled with gas chromatography mass spectrometry (SPME-GC/MS) will be applied for isolation and identification. For quantification of the volatile components the internal standard 2-nonanal was used. The effect of two enzymes "Endozym Aromatic" and "AR 2000" on the volatile profile of white and red wines from Macedonia will be examined.

For this purpose nine white wines "Temjanika and Muscat" produced from the most abundant white grape variety in Macedonia "Muscat de Frontignan" were selected. Due to the large abundance of bond volatile compounds, during wine-making procedure the enzyme "Endozym Aromatic" was applied. One of the targets of this PhD work was to examinate the volatile profile of enzymatically treated "Temjanika"wines.

"**Muscat de Frontignan"** is perhaps the oldest known and the most abundant white grape variety in Macedonia known as "Temjanika". It is one of several related varieties of Muscat. It most likely originated in Ancient Greece and expanded into Italy and France in Roman times. There is a record of its export from the French port of Frontignan during the

region of Charlemagne, well over 1100 years ago. Muscat de Frontignan has a wide geographical distribution but is often grown in small quantities. It grows in Mediterranean France, Greece, Hungary, Italy, Spain, Argentina, South Africa, Australia, and the United States, especially California. It is also grown in the Republic of Macedonia (Tikveš, and Skopje wine-growing region). The cluster of "Muscat de Frontignan" is small or middle large, with cylindric or cylindric-conical shape, medium filled or compact, and berries are small with round shape. The skin is thin, transparent, with green-yellow color (Fig.10). The wines have an intense, transparent, golden yellow color and aroma of grapefruits, pineapple, banana and melon (Bozinović, 1996).

One of the wine samples, which was object of the study in this PhD thesis is produced from Muscat Ottonel grape variety, the least abundant variety of white grapes produced in the territory of Macedonia. The volatile compounds responsible for the overall taste of the wine in Muscat Ottonel grape variety are predominantly in bond form as well as in Muscat de Frontignan grape variety. However, during the wine-making process this wine was not treated with enzymes. The goal of this part of PhD thesis is to examine the effect of enzyme "AR 2000" on the volatile profile of Muscat Ottonel wine in order to liberate the glucosidically bond volatile compounds responsible for the overall flavor of the wine.

"**Muscat Ottonel"** was cultivated for the first time in Angers in 1852. Bunches are small to medium, cylindrical with medium-sized, spherical berries, clear yellow with a delicately musky flavour (Fig. 10).

Fig. 10. Muscat de Frontignan (Temjanika; Wines of Macedonia, 2011) and Muscat Ottonel grape varieties

(With kind permission by Dr. J. Schmid, Geisenheim Research Center)

Alexandria Riesling wine was a mixture of different Muscat varieties and its wine-making procedure is unknown but, any enzymatic treatment is not applied during vinification.

Furthermore, Bistro Bianco wine was produced from "Chardonnay" grape variety without addition of any enzyme for glucosidically breakage of bond volatile compounds.

"Chardonnay" is a widely cultivated variety in the world, originating from France, from the Champagne and Burgundy regions. The cluster of "Chardonnay" is small or middle large, with cylindric or cylindric-conical shape, medium filled or compact, and berries are small with round shape. The skin is thin, transparent, with green-yellow color (Fig. 11). For cultivation, this variety needs middle fertile, deep and temperate moist soils. It is particulary sensitive to drought and highly resistant to low winter temperature. Chardonnay wines have intese, transparent, golden yellow color and fruity aroma.

Fig. 11. Chardonnay grape variety (Wines of Macedonia, 2011)

The volatile profile of three the most famous red wines was object of study in this PhD work. The most famous red wine from Macedonia "T' ga za Jug" is produced from Vranec grape variety. During its wine-making procedure the enzyme treatment was not included as well as in the vinification procedure of the remaining two examinated red wines produced from "Merlot" and "Cabernet Sauvignon" grape variety.

Appart from the terpenes as the most dominant volatile compounds in wines produced from Muscat varieties, the last goal of this PhD work is to find relationship between the effect of enzyme "AR 2000" on the alcohols, esters and fatty acids as the most abundant volatile compounds in red wines.

2. THEORETICAL PART

2.0. Countercurrent chromatography

Countercurrent chromatography refers to the process of liquid-liquid chromatography carried out without the addition of solid stationary phase for retention of stationary liquid components in chromatographic column (Berthod, 1991; Conway, 1991).

The term "countercurrent chromatography" usually defines processes in which one liquid phase is maintained by gravitational or centrifugal forces, distributed longitudinally into the column while a second immiscible liquid phase passes through stationary liquid phase usually in opposite direction relative to the column. The less dense phase replaces the heavier phase towards the tail, but the orientation is also known to be influenced by viscosity and interfacial tension. The separation process is achieved by two immiscible liquid solvents.

The choice of immiscible solvent systems depends largely on the matrix from which the desired compound is isolated. In order to select appropriate phases, the sample is dissolved in two immiscible solvent systems. The success of the separation process is predicted by concentration of the analytes in each of the phases by spectrophotometry, gas chromatography or thin-layer chromatography (Ito, 2005; Winterhalter, 2007). The general rule is choosing the solvent or mixture of solvents with higher density as mobile phase (lower phase) and solvent or mixtures of solvents with lower density as stationary phase (upper phase) in the elution mode "*head-to-tail*". In the opposite situation, when the solvent system with lower density is chosen as mobile phase, the "*tail-to-head*" mode is recommended. Simple matrices separation and isolation of compounds can be done with

two immiscible solvent systems such as hexane-methanol. But, in most cases the sample matrixes are complex and the great advantage of CCC is the possibility of using any liquid matrix that forms two immiscible phases (Ito, 2005).

During CCC separation the coils are rotated around their own "planetary" axis and simultaneously around the "solar" axis with velocities of 800-1000 rpm (Ito, 2005). During the separation process five steps are occurring: (i) the first step is loading of the coils with stationary phase by using a HPLC pump, (ii) dissolving the sample in equal volumes of both phases and its injection into CCC system, (iii) start rotation with 800 rpm approximately by pumping of the mobile phase, (iv) equilibration of the system by replacement of stationary with mobile phase and finally, (v) in the last step elution of the compounds from the mixture according to their coefficient values (Ito, 2005; Winterhalter, 2007).

The "CCC column" is usually a continuous open tube coiled on a spool which is rotated in centrifuge. Fig. 12 (A) depicts the distribution of the two mobile phases in the rotating coil. The spiral column is divided into two zones. The first "mixing" zone occupied one quarter of the area near to the centre of rotation and the rest of the area belonged to other "settling" zone. Fig. 12 (B) presents the spiral column which was stretched and arranged (position I to IV) for detailed description of the motion of the mixing zone along the column. More precisely, the picture below shows the mixing zone which has been passed through the spiral column with a rate of one round per one revolution. The solute inside the coil has been mixed by speed of 13 times per second at 800 rpm (Ito, 2005).

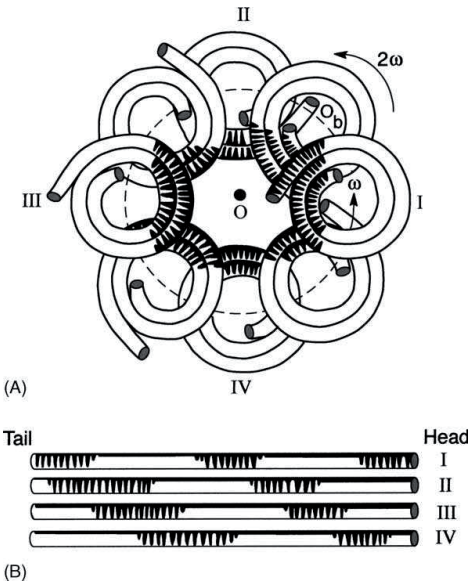


Fig. 12. The principle mechanism of separation by countercurrent chromatography (Ito, 2005)

Number of the coils in CCC system has a significant effect on the separation process. Results from analyses of Degenhardt *et al*. (2000a) indicated that a single coil CCC system was less efficient compared with a three coil system.

2.1. Application of countercurrent chromatography for isolation of grape pigments

The main advantage of countercurrent chromatography is the possibility of isolation of pure compounds from complex natural mixtures.

Isolation of polyphenols from food, especially natural pigments from grapes, wines and their by-products is important for replacement of synthetic colorants in food.

Countercurrent chromatography was widely used for isolation of anthocyanins from grapes and wines (Winterhalter, 2007; Degenhardt *et al*., 2000b; Schwarz *et al.*, 2003). According to the published results very polar solvent systems were used. The phases consisted of *n*-butanol as very polar solvent acidified with trifluoroacetic acid which acts as counterion for the flavylium ion (Fig. 15). The mode of separation was "*head-to-tail*" by using the phase with lower density as stationary phase (Degenhardt *et al*., 2000b; Schwarz *et al*., 2003).

Better resolution during the separation process was obtained by purification of the sample with XAD-7 resin in comparison with direct CCC separation of the crude anthocyanin extract (Schwarz *et al*., 2003).

The quantity of isolated and purified pigments depends on the plant material from which the isolation was performed. More than 700 mg of pure malvidin-3-glucoside was obtained from red wine in the work of Degenhardt *et al*. (2000a). Separation of anthocyanins glucosides were significant improved in the work of Schwarz *et al*. (2003).

In the work of Vidal *et al*. (2004) grape anthocyanins from rosé grape pomace and grape skins were fractionated by multilayer coil countercurrent chromatography (MLCCC) (Vidal *et al*., 2004). For separation and fractionation of anthocyanins the solvent system composed of *tert*-butyl methyl ether/*n*-butanol/acetonitrile/water acidified with trifluoroacetic acid (2/2/x/5 with x varying between 0.1 and 2.5) was used. During the separation process malvidin *p*-coumaroylglucoside was the most abundant

pigment with percentage of 60 % of total coumarates. Malvidin and peonidin-3-caffeoylglucoside were present in amounts up to 8 % in the same fraction (Vidal *et al*., 2004).

The chemical structure of anthocyanins is a flavylium cation which includes two benzene rings connected by an unsaturated cationic oxygenated heterocycle, derived from the 2-phenyl-benzopyrylium nucleus (Fig. 13). Five anthocyanidins have been identified in grapes and wines, with two or three substituents (OH and OCH_3) according to the substitution of the lateral nucleus (Ribéreau-Gayon & Glories, 2006). These molecules are much more stable in glycosidic form (anthocyanin) than in aglycone (anthocyanidin) form. Only monoglucosidic anthocyanins and acylated monoglucoside anthocyanins have been identified in *Vitis vinifera* grapes and wines. Acylation is made with *p*-coumaric caffeic or acetic acid as shown in Fig.14.

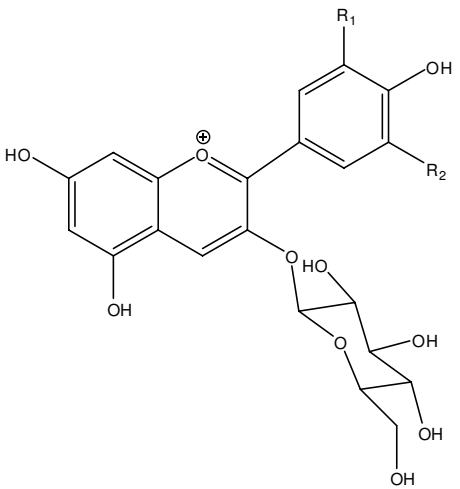


Fig. 13. Structure of anthocyanin-3-*O*-glucoside

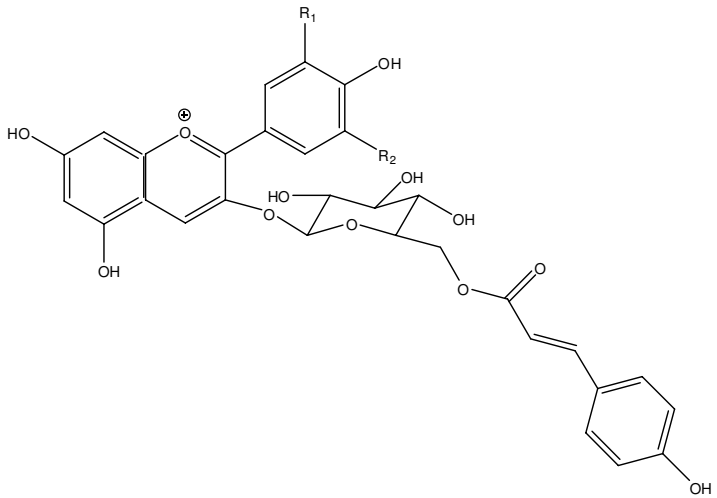
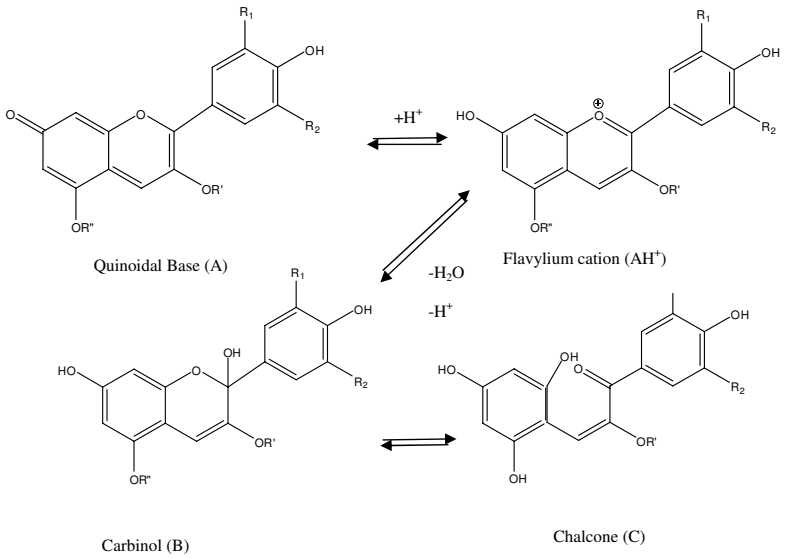


Fig. 14. Structure of anthocyanin-3-*p*-coumaroylglucoside

The most abundant anthocyanins in red grapes are delphinidin (3,5,7,3',4',5'-hexahydroxyflavylium), cyanidin (3,5,7,3',4'-pentahydroxyflavylium), petunidin (3,5,7,3',4'-pentahydroxy-5'-methoxyflavylium), peonidin (3,3',4'5'7-pentahydroxy-3'-methoxy-flavylium) and malvidin (3,5,7,4'-tetrahydroxy-3',5'-dimethoxyflavylium). The percentage of each anthocyanidin is influenced by cultivar type and viticultural conditions. Blueness of the grape is enhanced with increasing number of free hydroxyl groups. On the other hand, the redness color of the grapes is induced by methylation of the hydroxyl groups (Ribéreau-Gayon & Glories, 2006).

The grape berry appears purple in color because of hydrogen bonding between the flavylium cation and the quinoidal base (cf. Fig. 15). This combines a red form with a blue form, leading to purple. This hydrogen bonding is stabilized during thermovinification, giving those wines their

characteristic intensely purple color (Brouillard *et al*., 2003). In must, because of the low pH, when the anthocyanin dimer dissociates, the quinoidal base is converted to either a carbonyl pseudobase or a flavylium cation and the blue color is lost leaving a more intense red color Fig. 15 (Boulton, 2001).



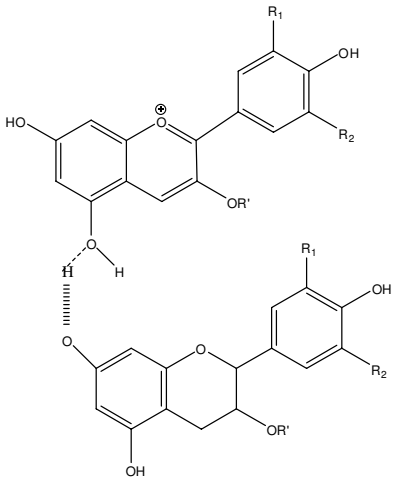


Fig. 15. Molecular transformation of anthocyanins in aqueous solution under varying pH and hydrogen bonding between the flavylium cation and the quinoidal base (Nikkhah *et al*., 2007)

Anthocyanin synthesis becomes pronounced after *véraison*. The timing and degree of anthocyanin synthesis depends on a variety of factors, such as temperature, light exposure, water status, sugar accumulation, and genetic factors. After reaching a maximum (usually at grape maturity), anthocyanin concentration tends to decline slightly (Jackson, 2008).

Biosynthetic pathway of anthocyanins is shown in Fig. 16. Chalcone synthase (CHS) catalyzes condensation of three acetate units from malonyl CoA with *p*-coumaroyl-CoA forming tetrahydroxychalcone. In the next step chalcone isomerase (CHI) catalyzes isomerization of the yellow-colored tetrahydroxychalcone to the colorless naringenin. Flavanon-3-hydroxylase (F3H) is converting the naringenin to dihydrokaempferol (DHK). Furthermore, dihydrokaempferol can be hydroxylated by flavonoid-3'-hydroxylase (F3'H) to produce dihydroquercetin (DHQ) or by 3'5'-hydroxylase (F3'5'H) to produce dihydromyricetin (DHM).

Enzymatic reactions are necessary for converting the dihydroflavonols DHK, DHQ, and DHM to anthocyanins. Dihydroflavonol 4-reductase (DFR) is converting the dihydroflavonols to flavan-3,4-*cis*-diols (leucoanthocyanidins) by reduction. Further oxidation, dehydratation, glycosylation of the different leucoanthocyanidins produces the corresponding brick-red pelargonidin, red cyanidin and blue delphinidin pigments (He *et al*., 2010).

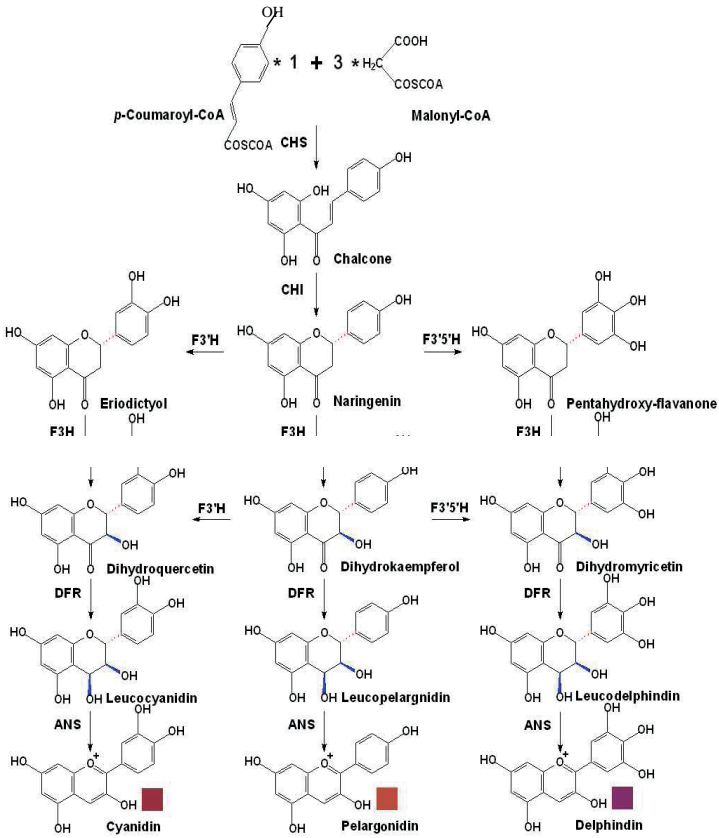


Fig. 16. The basic flavonoid pathway leading to the biosynthesis of colored anthocyanidins in grapes. **CHS**, chalcone synthase; **CHI**, chalcone isomerase; **F3H**, flavanone 3β-hydroxylase; **F3'H**, flavonoid 3'-hydroxylase; **F3'5'H**, flavonoid 3',5'-hydroxylase; **DFR**, dihydroflavonol 4-reductase; **ANS**, anthocyanidin synthase (He *et al.*, 2010)

The second part of flavonoid pathway (cf. Fig. 17) is starting after formation of anthocyanins by activity of anthocyanidin synthase (ANS).

Glycosylation is a very important modification because it is increasing the stability and hydrophilicity of anthocyanins. Anthocyanin glycosyltransferases catalyse the *O*-glycosylation which recognizes anthocyanins as sugar receptors. The process is activated with flavonoid glucosyltransferase (UFGT). In *Vitis vinifera* grapes, anthocyanidins can only be *O*-glycosylated at the C3 position with the addition of glucoses by the activity of UFGT. The highest activity for the UFGT enzyme was recommended for cyanidin as acceptor, but it can also use delphinidin as well as pelargonidin, peonidin, petunidin, and malvidin at lower levels at its optimal pH 8.0 (He *et al*., 2010).

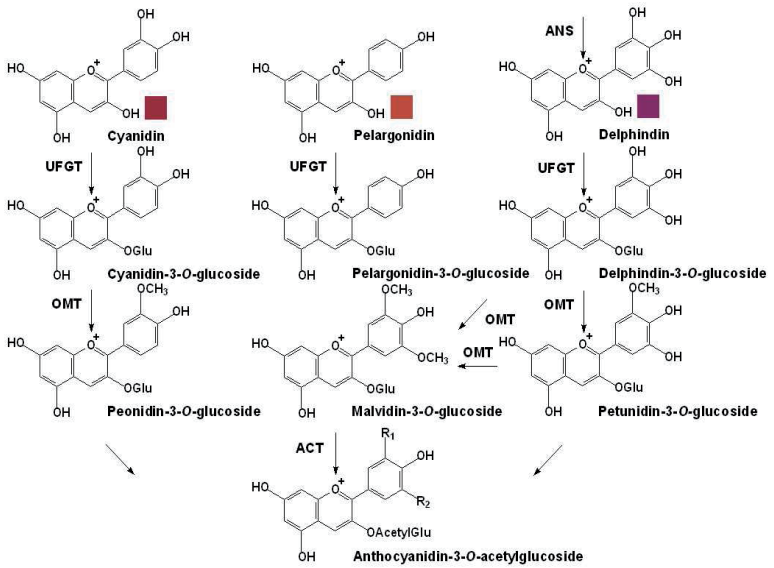


Fig. 17. The specific pathway for the anthocyanin modification of free anthocyanidins in grapes. **UFGT**, flavonoid glucosyltransferase; **OMT**, *O*-methyltransferase; **ACT**, anthocyanin acyltransferase (He *et al*., 2010).

2.2 Stilbenes in red wines

Stilbenes are phytoalexines which are synthesized as a response to localized stress such as infection by pathogenes (e.g. *Botrytis cinerea*) injuries or UV irradiation. The major phytoalexine is resveratrol and its *β*-glucoconjugated form which is known as piceid. Resveratrol and piceid are concentrated on the grape skins and exist in two isomeric forms *trans* and *cis*.

Biosynthetic pathway of stilbenoids is in common to flavonoids. Regarding the one of the explanations, *p*-coumaroyl coenzyme A (CoA) and malonyl CoA are main precursors of resveratrol in molar ratio 1:3. Malonyl CoA is produced from elongation of acetyl CoA units. In addition, the source for synthesis of *p*-coumaroyl CoA is phenylalanine derived from sugars via the shikimate pathway. By oxidative deamination phenylalanine is converting to cinamic acid which is hydroxylated enzymatically to *p*-coumaric acid. The condensation of *p*-coumaroyl CoA with three molecules of malonyl CoA is producing *trans*-resveratrol by activity of stilbene synthase (Waffo-Teguo *et al*., 2008).

The other explanation is beginning during the process of cyclization of a steryl-3,5,7-triketoheptanoic acid when two main routes are occurring. The first main route presents C-acylation which produces a calcone as intermediary compound and the process is ending by formation of flavonoids (cf. Fig. 18). The second route of biosynthetic pathway consists of a present aldol condensation of the same intermediate polyketide which produces a stilbene-2-carboxylic acid (Rentzsch *et al*., 2008).

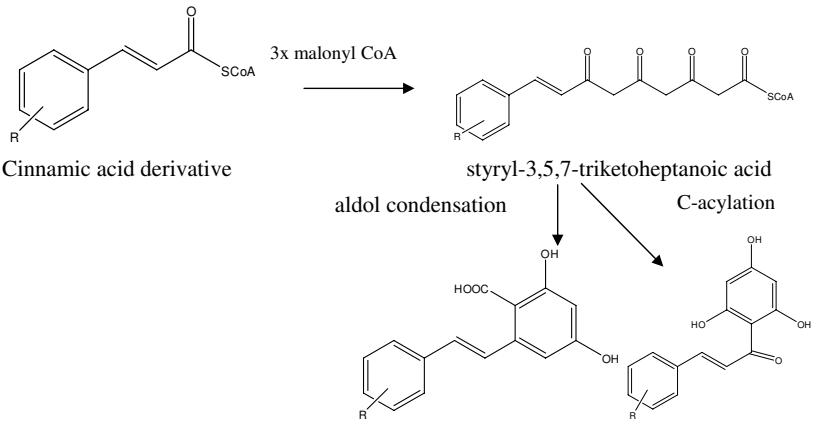
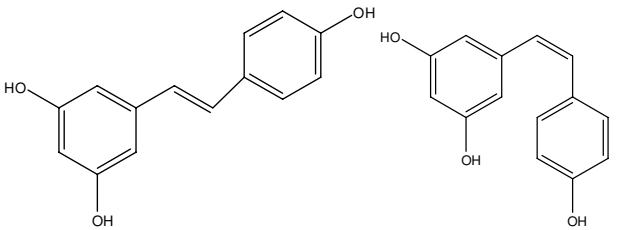
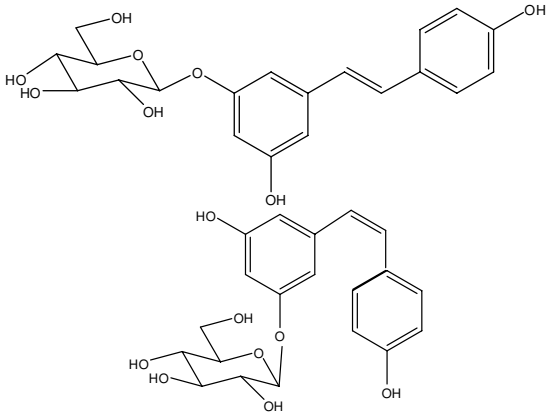


Fig. 18. Biosynthetic pathway of stilbenoids and flavonoids (Rentsch *et al*., 2008)

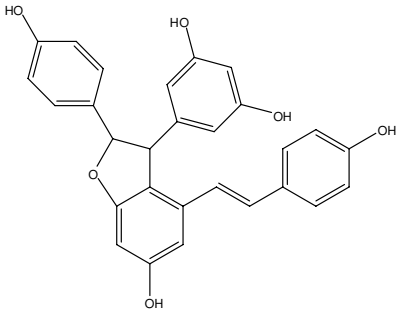
The concentration of resveratrol and piceid in wines depends on many factors, such e.g. the level of fungal infections, particularly *Botrytis cinerea*. Generally, red grapes have higher concentration of stilbenes than white grapes (Ribéreau-Gayon & Glories, 2006)**.** Fig. 19 shows the chemical structure of the most abundant monomeric and dimeric stilbenes present in wines.



trans- and cis- forms of 3,5,4'-hydroxystilbene (resveratrol)



trans- and cis-resveratrol-3-*O*-*β*-glucopyranoside (piceid)



ε-viniferin

Fig. 19. Structures of the most abundant monomeric and dimeric stilbenes in red wines

2.3. Volatile compounds in wines

Fig. 20 presents chemical structures of the most important monoterpenes in wines. Monoterpenes are the most abundant volatile compounds in Muscat wines. However, they are only partially responsible for the overall flavor of the wines.

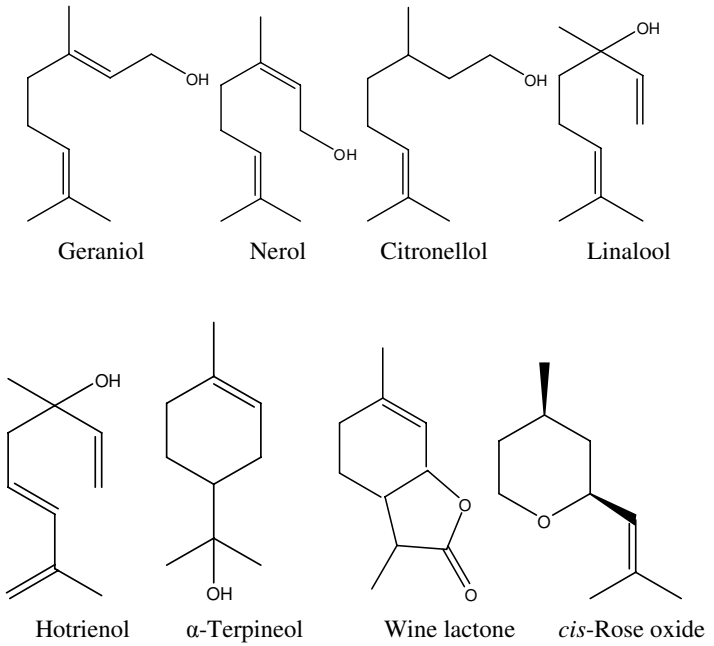


Fig. 20. Chemical structures of the most abundant monoterpenes in wines (Moreno-Arribas & Polo, 2009)

There are three possible biosynthetic pathways of terpenes formation. The first pathway presented on Fig. 21(a) is occurring in cytoplasma of grapes and it is known as Mevalonic acid pathway (MVA). During the (MVA) pathway a molecule of acetyl-CoA undergoes a Claisen condensation with a molecule of malonyl CoA forming acetoacetyl CoA. An Aldol condensation with third molecule of malonyl CoA transforms the acetoacetyl CoA into 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA). Reduction with HMG-CoA reductase yields mevaldic acid which is reduced again to mevalonic acid. After three consecutive phosphorylations, labile intermediate compound 3-phospho-5-pyrophosphomevalonate is

transformed to 3-isopentenyl pyrophosphate (IPP) as a precursor of sesquiterpene, triterpene and polyterpene (Wüst, 2003).

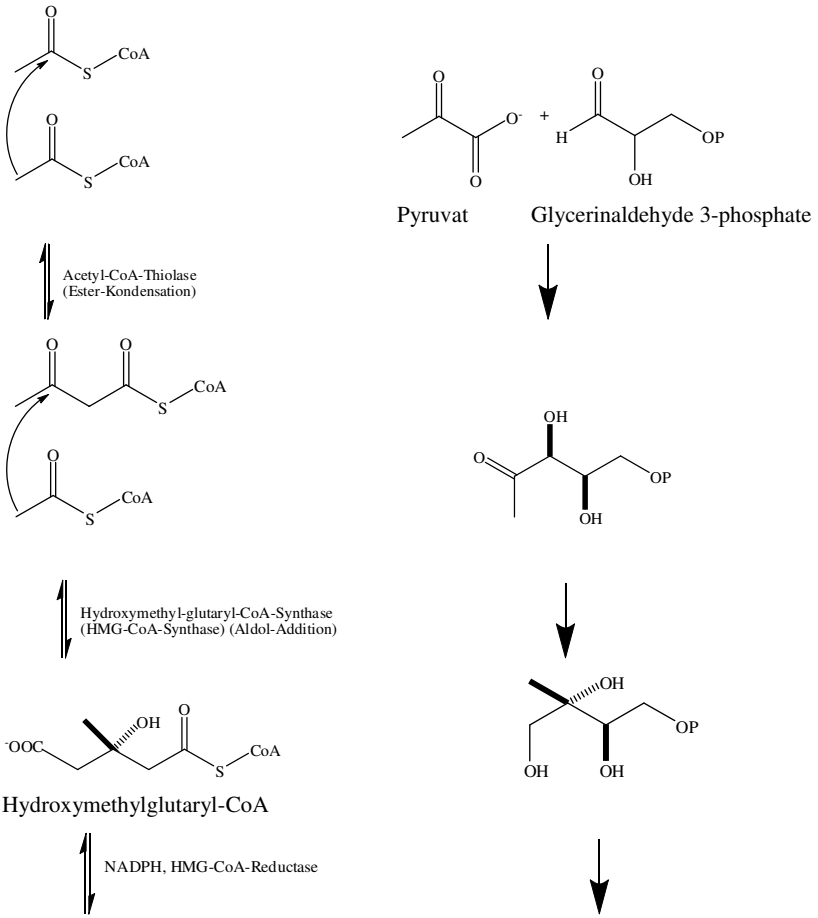


Fig. 21.(a) Mevalonic acid pathway (MVA) (Wüst, 2003)

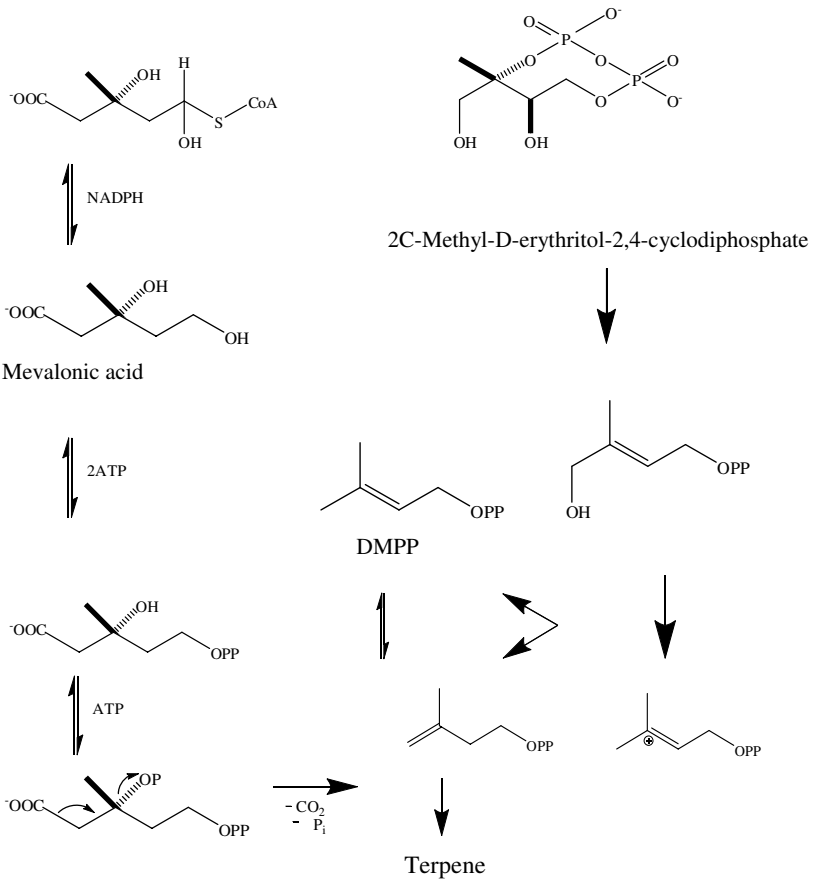
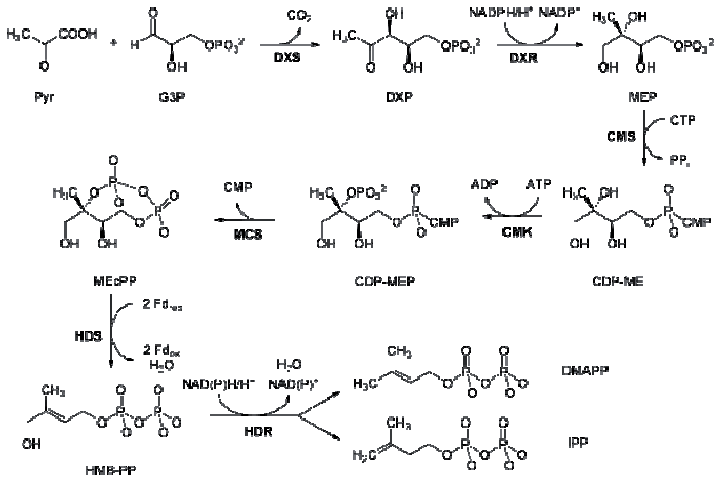


Fig. 21.(a) Mevalonic acid pathway (MVA) (Wüst, 2003) (Cont'd)

The second pathway of isopentenyl pyrophosphate in grapes plastid (DOXP pathway) was discovered in 2002. It starts with formation of 1-deoxy-D-xylulose 5-phosphate (DXP) from pyruvate and glyceraldehydes 3-phosphate. After the rearrangement to 2-C-methyl-D-erythritol 4-phosphate (MEP) and serial conversions the final product of this pathway is

again isopenthyl diphosphate (IPP) as a precursor of monoterpene, diterpene and carotinoide compounds in grapes (Wüst, 2003).



Monoterpenes, Diterpenes, Carotenoids

Fig. 21.(b) DOXP pathway (Wikipedia)

The third possible biosynthetic pathway depicted in Fig. 22 is starting from dimethylallyl pyrophosphate as precursor for geranyl diphosphate. The final products are citronellol, α-terpineol and *trans*-furan linalool oxide (Moreno-Arribas & Polo, 2009).

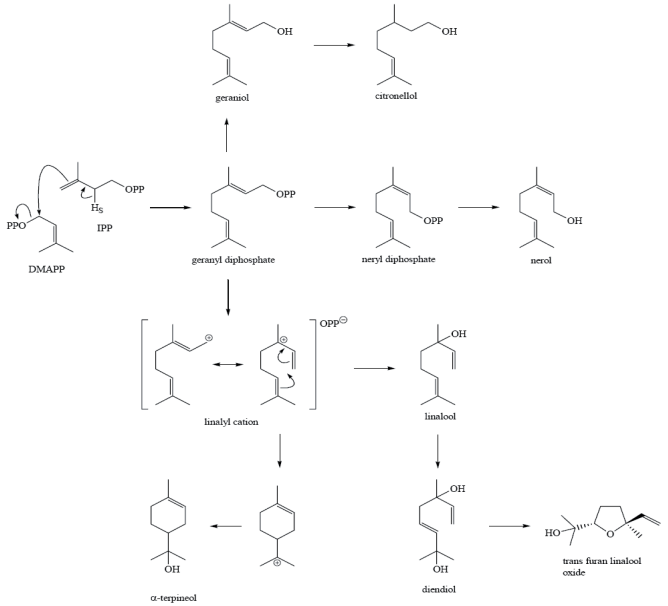


Fig. 22. Biosynthesis of the most abundant monoterpenes in wines (Moreno-Arribas & Polo, 2009)

Except terpenes, the second important class of chemical compounds responsible for the smell and the taste of the wines are volatile esters. Two main types of esters are presented in the wine: ethyl esters of fatty acids and acetate esters. Fatty acid ethyl esters (ethyl butanoate, ethyl hexanoate and ethyl octanoate) are formed during alcoholic fermentation from AcylCoA intermediates (Fig. 23).

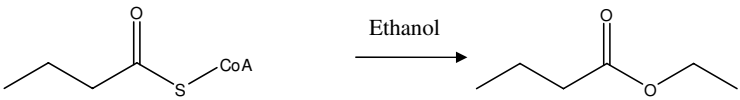


Fig. 23. Formation of fatty acid ethyl esters

The acetate esters (hexyl acetate, isoamyl acetate) are formed during reaction between AcetylCoA and higher alcohols which are degradation products of amino acids. Hexanol as a major alcohol in wines is an oxidation product of linoleic acid during crushing (Fig. 24).

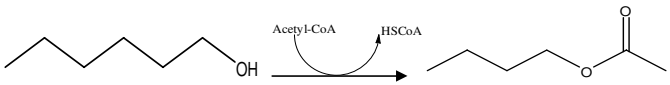


Fig. 24. Formation of acetate esters

Enzymatic hydrolysis of esters also occurs during fermentation (via esterases) while chemical hydrolysis occurs during storage and aging.
Generally, the process of formation of the most important volatile compounds during alcoholic fermentation by application of *Saccharomyces cerevisiae* yeasts discussed previously is presented in Fig. 25.

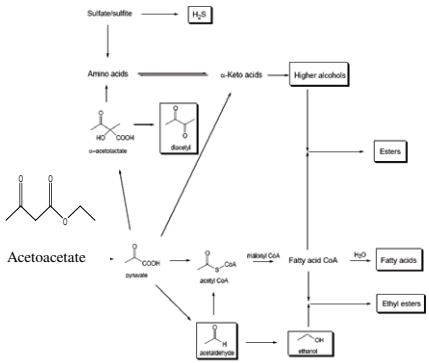


Fig. 25. Formation of volatile compounds during yeast fermentation

Carotenoids such as β-carotene, lutein, neoxantin and violaxantin are instable compounds because of many double bonds in their structure. These compounds are precursors for nor-isoprenoids as very important flavor compounds especially for bottle aged wines. Carotenoids play role in

many enzymatic reactions generating aromatic compounds responsible for the taste of the grapes and wines. The carbonyl compounds which are formed by breakage of carotenoids structure can consist of 9, 10, 11 and 13 carbon atoms (Wüst, 2003). The possible fragmentation of carotenoid structure and releasing of carbonyl compounds is depicted in Fig. 26.

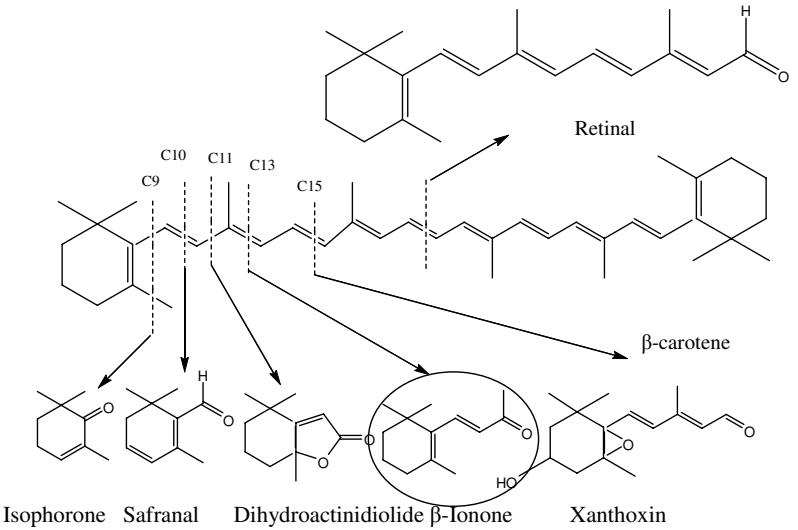


Fig. 26. Formation of carbonyl compounds by carotenoids degradation (Wüst, 2003)

The compounds with significant influence on the flavor of the wines are C_{13}-norisoprenoids. β-Damascenone, β-ionone, 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN), 2,2,6-trimethylcyclohexanone (TCH) and vitispirane have significant sensorial impact on wine flavor because of their low perception thresholds. Their concentrations can be markers for quality of the wines. Therefore, many studies were subjected to norisoprenoids and their effect on wines. The relationship between the

levels of norisoprenoids in grapes and wines was examined in the work of Ristic *et al*. (2010). The increased concentration of glucosylated precursors to β-damascenone in Shiraz grape berries had been connected to increased sunlight penetration to the fruits in the work of Bureau *et al*. (2000).

The role of norisoprenoids in Riesling wine after liberation from their precursors was discussed in the work of Winterhalter *et al*. (1990a; 1990b). Identification of the two glycosidically bound precursors of 1,1,6-trimethyl-1,2-dihydronaphthalene (TDN) and its possible biosynthetic pathway was discovered by working group of Winterhalter (Winterhalter *et al*., 1991).

In summary, soil and sunlight conditions, varieties of grapes, vinification procedures and aging of the wines are the most important factors determining the norisoprenoid contribution to the aromatic profile in wines.

3. RESULTS AND DISCUSSION

3.1 Occurrence of resveratrol in Vranec and Merlot wines from Macedonia: effect of variety and enological practices

In this chapter the influence of variety and enological practices on the concentration of stilbenes in red wines produced in the experimental laboratory at the Institute of wine-making in Skopje will be described.

The concentration of *trans*-resveratrol and *trans*-piceid was determined in 12 Vranec and 12 Merlot wines (Table 1 and Table 2) by direct injection of the wines into HPLC. The effect of variety and enological practices, or more precisely, the effect of maceration time of 3, 6 and 10 days, application of Macedonian yeast "Vinalco" and French yeast "Levuline CHP" and concentration of SO_2 of 30 and 70 mg/L on the concentration of *trans*-resveratrol and *trans*-piceid was object of study in this chapter. The chromatograms of *trans*-resveratrol and *trans*-piceid of Vranec and Merlot wine are shown in Fig. 27-28.

3. Results and Discussion

Table 1. Concentration of *trans*-piceid in Vranec wines

sample	*days of maceration*	*type of yeast*	*Conc. of SO_2 mg/L*	*trans-piceid* mg/L*
V11	3	Macedonian	30	0.23±0.00
V2	3	French	30	0.56±0.00
V3	3	Macedonian	70	0.13±0.02
V4	3	French	70	0.20±0.09
V5	6	Macedonian	30	0.94±0.01
V6	6	French	30	1.49±0.00
V7	6	Macedonian	70	1.48±0.00
V8	6	French	70	1.49±0.01
V9	10	Macedonian	30	0.87±0.00
V10	10	French	30	1.58±0.05
V11	10	Macedonian	70	0.88±0.02
V12	10	French	70	2.24±0.08

*Concentrations are expressed in mg/L ± SD of two replicates.

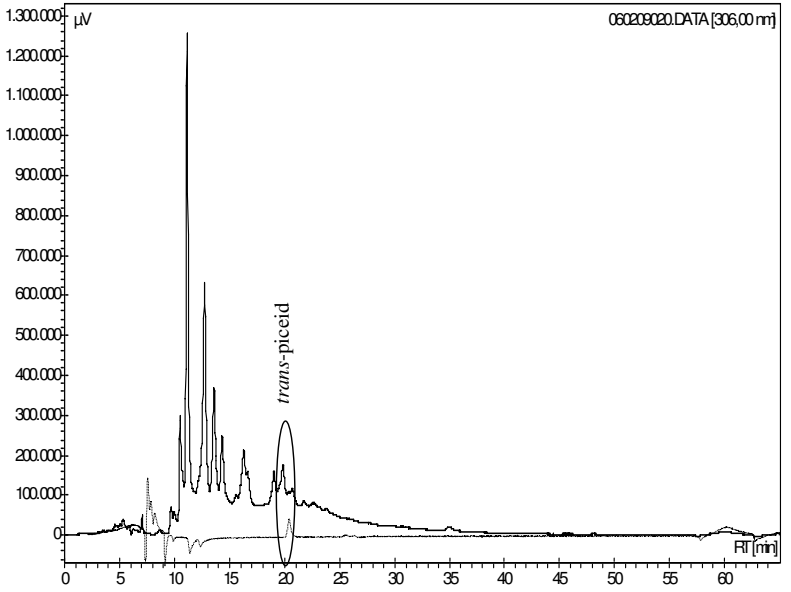


Fig. 27. Chromatograms of Vranec wine V9, with 30 mg/L SO_2, macerated for 10 days, fermented with Macedonian yeast "Vinalco" and standard of *trans*- piceid (---) at t_R =19.8 min. recorded at 306 nm

Table 2. Concentration of *trans*-piceid and *trans*-resveratrol in Merlot wines

sample	*days of maceration*	*type of yeast*	*Conc. of SO_2 mg/L*	*trans-piceid* mg/L*	*trans-resveratrol* mg/L*
M1	3	Macedonian	30	2.17±0.21	0.22±0.19
M2	3	French	30	2.91±0.07	0.81±0.07
M3	3	Macedonian	70	2.54±0.19	0.22±0.05
M4	3	French	70	2.75±1.00	0.30±0.10
M5	6	Macedonian	30	2.95±1.38	1.49±0.06
M6	6	French	30	3.83±0.21	1.22±0.09
M7	6	Macedonian	70	3.18±0.76	0.89±0.00
M8	6	French	70	4.10±0.86	0.00±0.00
M9	10	Macedonian	30	4.21±0.70	0.43±0.06
M10	10	French	30	4.65±0.46	1.43±0.19
M11	10	Macedonian	70	2.89±0.57	0.44±0.09
M12	10	French	70	4.48±0.18	1.75±0.21

*Concentrations are expressed in mg/L ± SD of two replicates.

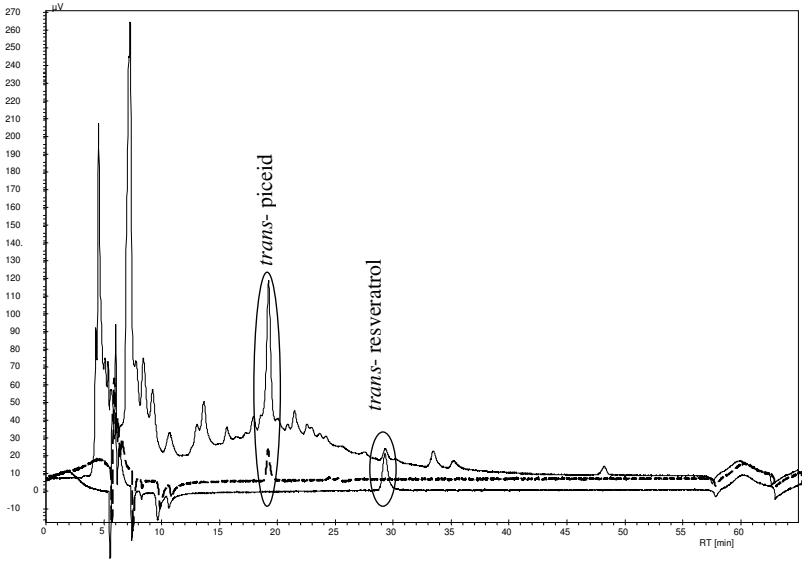


Fig. 28. Chromatograms of Merlot wine M1, with 30 mg/L SO_2, macerated for 3 days, fermented with Macedonian yeast "Vinalco", standard of *trans*- piceid (---) at t_R =19.8 min and standard of *trans*- resveratrol (—) at t_R = 29.8 min, recorded at 306 nm

3.1.1. Effect of variety

In Merlot wines both *trans*-resveratrol and *trans*-piceid were detected, whereas in Vranec wines only *trans*-piceid was present. As it is shown in Fig. 29, the contents of *trans*-piceid in Merlot wines were higher compared to Vranec wines obtained under the same vinification conditions. The biggest difference in the concentration of *trans*-piceid in favor of Merlot wines was detected in wines produced with 3 days of maceration time (V1M1-V4M4).

The aglycon form, i.e. free *trans*-resveratrol, is formed after enzymatic cleavage of piceid during the winemaking process (La Torre *et al*., 2004). Because of the low content of *trans*-piceid in Vranec wines, it was expected that it would not be possible to detect the aglycon in this variety.

Merlot wine M8 produced with 6 days of maceration using French yeast and 70 ppm SO_2 endows with high concentration of *trans*-piceid (4.10±0.86 mg/L) in comparison with other wines produced with 6 days of maceration time, but has no detectable concentration of *trans*-resveratrol.

Comparing the results of this study related to the concentrations of *trans*-resveratrol in Merlot and Vranec wines from Macedonia and those published for Merlot wines from different regions of the world, it can be concluded that Macedonian Merlot and Vranec wines have lower concentrations of *trans*-resveratrol. Nevertheless, the concentrations are similar to *trans*-resveratrol in Merlot wines from Japan (0.6-2.1 mg/L), USA (0.4-2.7 mg/L), Greece (n.d.-2.5 mg/L), China (n.d.-3.2 mg/L), South America (0.8-2.2 mg/L) and Chile (0.8-1.6 mg/L) (Stervbo *et al*., 2007), red wines from Aragón (0.32-4.44 mg/L) (Abril *et al*., 2005), some red wines from the Galician region (Feijóo *et al*., 2008), red wines from Hungarian Vanálly region (0.2-3.2 mg/L) (Pour Nikfardjam *et al.,* 2006) and Merlot wines from Serbia (1,95 mg/L) (Atanacković *et al*., 2012). Regarding the level of *trans*-piceid in both varieties of red wines, it is similar to the levels reported for red wines from Japan (0.17-3.54 mg/L) and USA (n.d-0.59 mg/L) (Stervbo *et al*., 2007). These concentrations are expected because of the similar climatic conditions with approximate temperature in the range between 24-33 °C during summer period without rainfall.

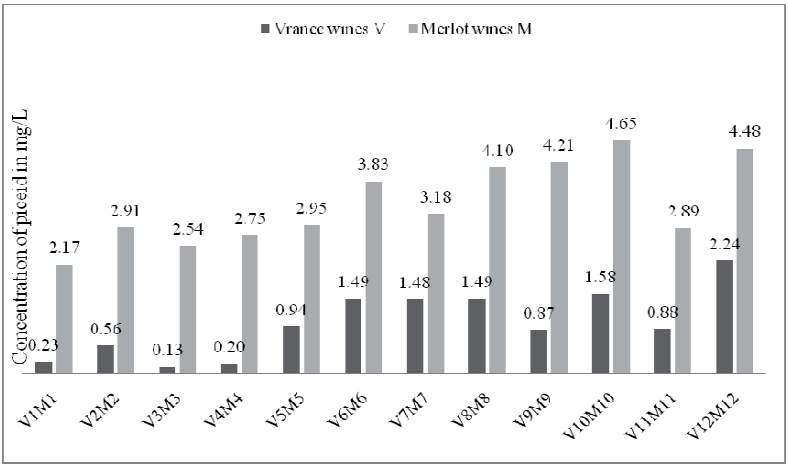


Fig. 29. Histogram of concentrations of *trans*-piceid in Merlot and Vranec wines produced under the same winemaking conditions

(for winemaking conditions cf. Table 1 and Table 2)

3.1.2. Effect of maceration time

Maceration time influences significantly the extraction of stilbenes from grapes into the wine. According to the results shown in Table 1 and 2, it can be noticed that the lowest concentration of *trans*-piceid was detected in Vranec wine V3 produced with a maceration time of 3 days, by adding 70 ppm of SO_2 and by using the Macedonian yeast "Vinalco". The results suggest that the concentration of resveratrol and piceid in 3 days of maceration time is very low, because of very short time of extraction and lower concentration of ethanol. Solubility of resveratrol and piceid is higher in ethanol, hence the extraction of both stilbenes is much efficient at the last stage of wine-making when the concentration of ethanol is higher due to the metabolic activity of yeasts. These results are in accordance with

the findings of Pezet & Cuenat, who explained significantly higher concentrations of resveratrol up to 6 days of maceration by the increased concentration of ethanol in wines (Pezet & Cuenat, 1996).

Prolongation of the extraction time increases the concentration of *trans*-resveratrol and *trans*-piceid. Consequently, the highest concentration of *trans*-piceid is detected in Merlot wine M10, produced with 10 days of maceration time using French yeast "Levuline CHP" and 30 ppm of SO_2 (Fig. 30). Similarly, the highest concentration of *trans*-resveratrol is detected in Merlot wine M12 with 10 days of maceration time using French yeast and 70 ppm SO_2 (Fig. 31).

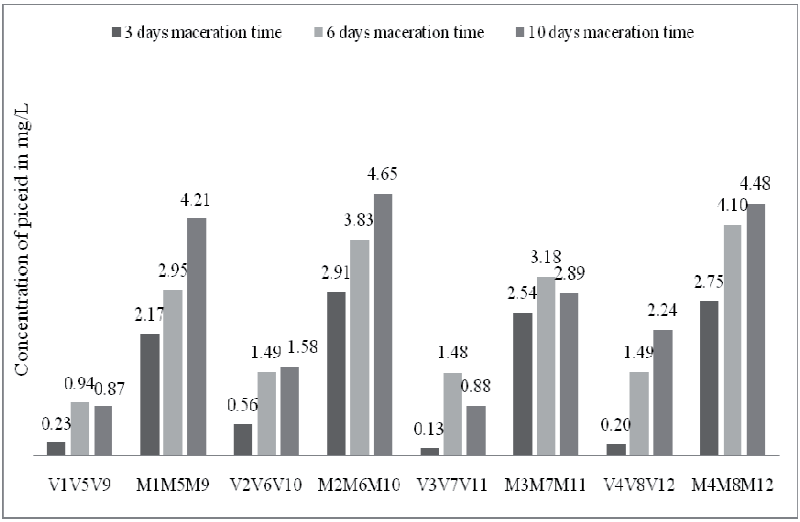


Fig. 30. Histogram of concentrations of *trans*-piceid in Merlot (M) and Vranec (V) wines in relation to the maceration time

(for further winemaking conditions cf. Table 1 and Table 2)

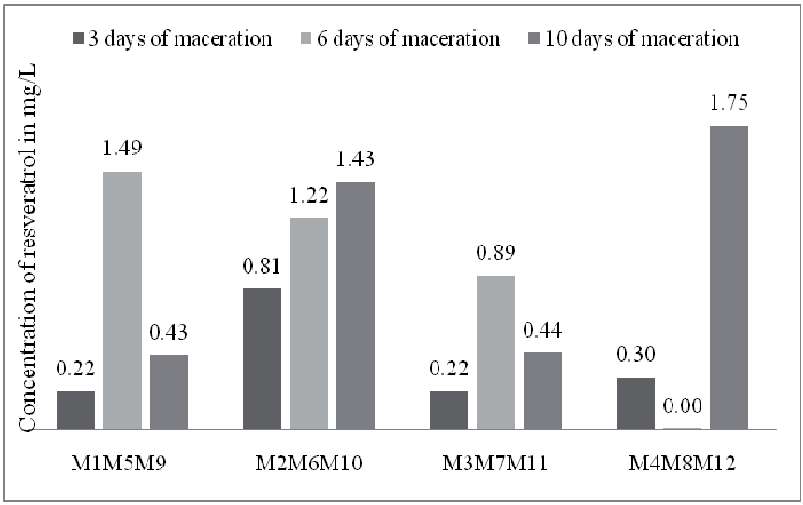


Fig. 31. Histogram of concentrations of *trans*-resveratrol in Merlot wines in relation to the maceration time (for further winemaking conditions cf. Table 1 and Table 2)

However, Merlot and Vranec wines with relatively low concentrations of *trans*-resveratrol and *trans*-piceid reached maximal concentrations of both stilbenes already after a shorter period (i.e. after 6 days of maceration). These results are in accordance with findings of Pezet & Cuenat and the research group of Vrhovsek, who stated that after five to six days of maceration the concentration of *trans*-piceid starts to decrease due to the enzymatic cleavage of the glycosidic bond (Pezet & Cuenat, 1996; Vrhovsek *et al*., 1997). Another possible explanation for decreasing levels of *trans*-resveratrol from 6 to 10 days of maceration time could be due to the yeast metabolism as occurs for other wine components such as anthocyanins, thiols or volatile phenols (Poussier *et al*., 2003). This behavior indicates the possibility of metabolization of resveratrol by yeasts.

The effect of maceration time on the level of concentration of *trans*-piceid is statistically significant (*p*-value is 0.027 for Merlot wines and 0.000 for Vranec wines). The results are presented in Table 3. Similarly, the effect of maceration time on the concentration of *trans*-resveratrol in Merlot wines is statistically significant (*p*-value is 0.000). Interactions between the two factors maceration time and type of yeasts (factor x_1x_2) and maceration time and concentration of SO_2 (factor x_1x_3) are also significant for *trans*-piceid in Vranec wines and *trans*-resveratrol in Merlot wines.

It is indicative that interaction between factors is significant only for *trans*-piceid in Vranec wines and *trans*-resveratrol in Merlot wines because those concentrations were much lower in comparison with concentrations of *trans*-piceid in Merlot wines. At the beginning of maceration when the concentrations of *trans*-piceid in Vranec wines and *trans*-resveratrol in Merlot wines were lower, the amount of SO_2 did not play a significant role in extraction. At the end of maceration when the concentration of both stilbenes is higher the effect of SO_2 is significantly stimulating extraction of *trans*-piceid from 1.58±0.05 mg/L in V10 to 2.24±0.08 mg/L in V12 wine. Regarding the interaction between maceration time and yeast, the difference between concentrations of *trans*-resveratrol in M9 and M10 wines and M11 and M12 wines is higher for 10 days than for 3 days of maceration.

3.1.3. Effect of yeast

All Vranec wines contain higher concentrations of *trans*-piceid after application of French yeast compared to Macedonian yeast (Fig. 32). The concentration of *trans*-piceid in V2 and V4 produced by application of

"Levuline CHP" is twice as big in comparison with wines V1 and V3 produced under the same vinification by using "Vinalco". The difference between the concentrations of *trans*-piceid in V9 and V10 is also twice as big as well as the difference in concentrations of piceid in V11 and V12 Vranec wines. This relationship is in correlation with results of the research group of Vacca, who explained that the difference in level of resveratrol in wines is a result of various types of yeasts applied during winemaking (Vacca *et al*., 1997).

A higher concentration of *trans*-piceid was also detected in Merlot wines produced with "Levuline CHP" compared to "Vinalco". However, the difference was not statistically significant as the *p*-values from General Linear Model for factor x2 (yeast factor) was 0.811. From the histogram in Fig. 32 it can be noticed that the concentration of *trans*-piceid did not increase rapidly because the free aglycon form started to increase from day 3 until day 10 of maceration time. This is in accordance with the explanation of the research group of Vrhovsek, suggesting that the highest *β*-glucosidase activity of yeast was observed after 4 days of maceration time when the concentration of resveratrol increases (Vrhovsek *et al*., 1997).

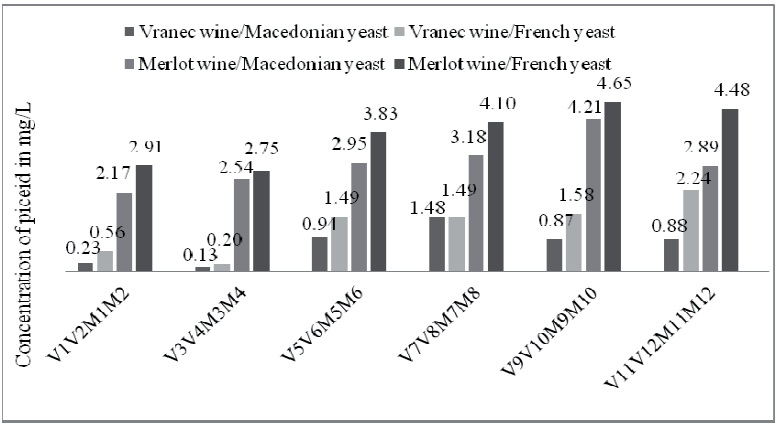


Fig. 32. Histogram of concentrations of *trans*-piceid in Merlot and Vranec wines produced with different yeasts

(for further winemaking conditions cf. Table 1 and Table 2)

Regarding *trans*-resveratrol levels in Merlot wines, the tendency of higher values for wines produced by "Levuline CHP" was not changed (cf. Fig. 33). The concentration of *trans*-resveratrol in Merlot wine M1 was 0.22±0.19 in comparison with the concentration of 0.81±0.07 mg/L in wine M2 produced under same winemaking process with different type of yeast. The concentration of *trans*-resveratrol in Merlot wines M10 and M12 produced with 10 days of maceration time and "Levuline CHP" is approximately four times higher in comparison to its concentration in corresponding wines M9 and M11 produced with Macedonian yeast "Vinalco". The statistical analysis indicates that the difference is statistically significant (*p*-value of 0.001). Moreover, the interaction between maceration time and yeast (factor x1x2) has also a significant effect (*p*-value of 0.000) since the difference between concentrations of *trans*-resveratrol in wines produced by two yeasts increases noticeably from day 3 to 10 of maceration time.

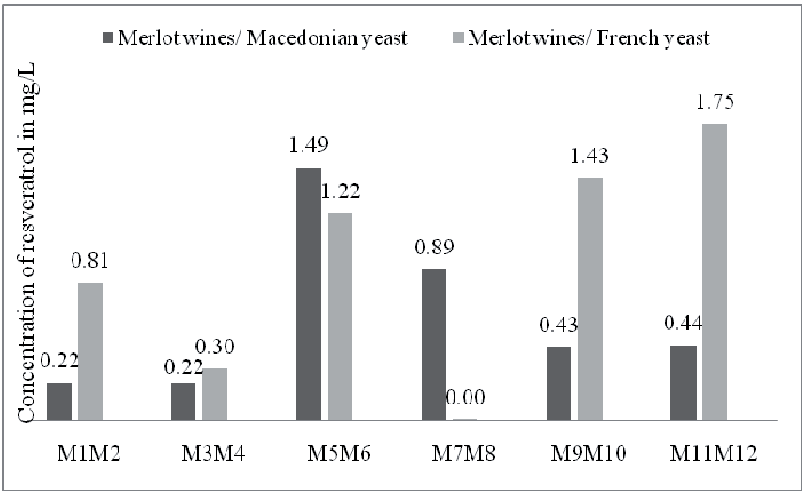


Fig. 33. Histogram of concentrations of *trans*-resveratrol in Merlot wines produced with different yeasts

(for further winemaking conditions cf. Table 1 and Table 2)

3.1.4. Effect of SO_2

SO_2 plays a role as antioxidant and antimicrobial agent during the winemaking process. Thus, it inactivates grape enzymes, such as polyphenoloxidases, protecting the polyphenols from oxidation and precipitation during the fermentation (Castellari *et al*., 1998).

The assumption that higher concentration of SO_2 will increase the concentration of *trans*-piceid and its aglycon form in wines is not true for all wines. According to the histograms in Fig. 34 it is obvious that this effect is true only for *trans*-piceid in Merlot wines for 3 and 6 days of maceration time, but the difference is statistically not significant. As far as *trans*-resveratrol is concerned, Fig. 35 indicates higher concentration of

trans- resveratrol for wines produced with 30 ppm SO_2 in comparison with those produced with 70 ppm SO_2. The difference is statistically significant as the *p*-value is 0.002.

The effect of SO_2 is in agreement with findings of the research group of Castelleri, who explained that SO_2 did not increase the extraction of resveratrol from the grapes, but reduced its oxidation during the prefermentative phase when the concentration of piceid and its aglycon is lower (Castellari *et al*., 1998).

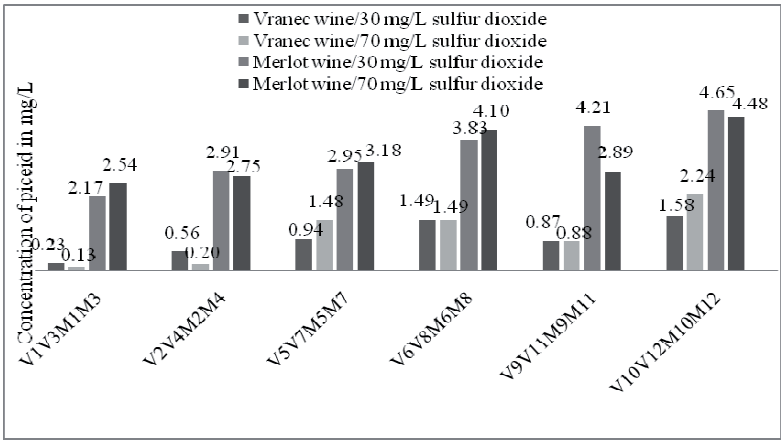


Fig. 34. Histogram of concentrations of *trans*-piceid in Merlot and Vranec wines produced with different concentration of SO_2

(for further winemaking conditions cf. Table 1 and Table 2)

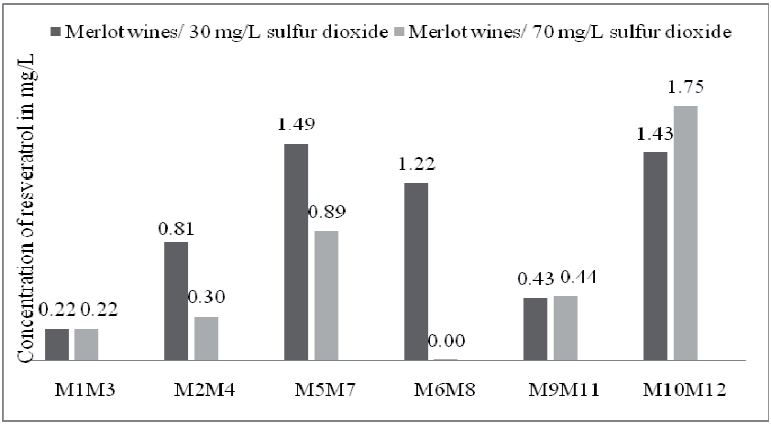


Fig. 35. Histogram of concentrations of *trans*-resveratrol in Merlot wines produced with different concentration of SO_2

(for further winemaking conditions cf. Table 1 and Table 2)

Table 3. Significant probability (*p*-values and *F*-ratio) of the independent variable effects (maceration time, type of yeast and SO_2 concentration level)

Responsible Variables	Main effects			Interaction effects		
	x_1	x_2	x_3	x_1x_2	x_1x_3	x_2x_3
***trans*-piceid** (Merlot wines)						
p-value	**0.027**	0.811	0.053	0.502	0.816	0.850
F-ratio	**4.71**	0.06	4.49	0.72	0.21	0.04
***trans*-resveratrol** (Merlot wines)						
p-value	**0.000**	**0.001**	**0.002**	**0.000**	**0.000**	0.115
F-ratio	**21.12**	**15.89**	**13.64**	**14.08**	**36.08**	2.83
***trans*-piceid** (Vranec wines)						
p-value	**0.000**	**0.000**	0.116	**0.001**	**0.016**	0.720
F-ratio	**95.68**	**45.76**	2.81	**12.79**	**5.62**	0.13

* Significant at $p < 0.05$

x_1, x_2 and x_3 represents the main or single effect of maceration time, type of yeast and SO_2 concentration level, respectively x_1x_2, x_1x_3 and x_2x_3 represents the interaction between maceration time and type of yeast, interaction between maceration time and SO_2 concentration level and interaction between type of yeast and SO_2 concentration level, respectively

3.2. Influence of winemaking process on the resulting antioxidant activity and sensory profile of Vranec and Merlot wines from Macedonia

In the following chapter the antioxidant activity of the wines examined in the first chapter regarding stilbenes content will be discussed.

The antioxidant activity of the twenty four wine samples from Vranec and Merlot grape variety obtained under different winemaking conditions, i.e. type of yeast (Macedonian "Vinalco" and French "Levuline CHP" yeast), different concentration of SO_2 (30 and 70 mg/L) and time of maceration (3, 6 and 10 days) were measured. The TEAC value of the wines expresses the concentration of a Trolox solution whose antioxidant activity is identical to that of the wine itself. It is obtained by interpolating the decrease in absorbance (corresponding to a five time diluted wine sample) on the calibration curve, thus obtaining a concentration of Trolox. Appropriate corrections were made taking the dilution into account. The TEAC values and standard deviation of measurements in duplicate are shown in Table 4 for Vranec wines and Table 5 for Merlot wines. The histograms of TEAC values are shown in Figures 36 and 37, respectively.

Table 4. The TEAC values of twelve wines from Vranec grapes obtained under different type of yeast (Macedonian and French yeast, "Vinalco" and "Levuline CHP", respectively), different concentration of SO_2, 30 and 70 mg/L, respectively and time of maceration 3, 6 and 10 days.

Sample	variety	Maceration time/days	type of yeast	conc. SO2/ppm	TEAC[a] 6 min
V1	Vranec	3	Macedonian	30	4.10 ±0.01
V2	Vranec	3	Macedonian	70	6.24 ±0.21
V3	Vranec	3	French	30	3.44 ±0.22
V4	Vranec	3	French	70	5.75 ±0.58
V5	Vranec	6	Macedonian	30	8.74 ±0.22
V6	Vranec	6	Macedonian	70	9.38 ±0.28
V7	Vranec	6	French	30	8.68 ±0.56
V8	Vranec	6	French	70	8.14 ±0.19
V9	Vranec	10	Macedonian	30	7.13 ±0.10
V10	Vranec	10	Macedonian	70	10.03 ±0.17
V11	Vranec	10	French	30	9.94 ±0.07
V12	Vranec	10	French	70	10.99 ±0.74

[a]Expressed as mmol Trolox per liter. Each value corresponds to the mean and standard deviation of two repetitions ±sd.

Table 5. The TEAC values of twelve wines from Merlot grapes obtained under different type of yeast (Macedonian and French yeast, "Vinalco" and "Levuline CHP", respectively), different concentration of SO_2, 30 and 70 mg/L respectively and time of maceration 3, 6 and 10 days.

Sample	variety	Maceration time/days	type of yeast	conc. SO_2/ppm	TEAC[a] $_{6\ min}$
M1	Merlot	3	Macedonian	30	6.66 ±0.50
M2	Merlot	3	Macedonian	70	6.88 ±0.00
M3	Merlot	3	French	30	4.72 ±0.01
M4	Merlot	3	French	70	5.36 ±0.21
M5	Merlot	6	Macedonian	30	6.25 ±0.07
M6	Merlot	6	Macedonian	70	6.60 ±0.12
M7	Merlot	6	French	30	5.60 ±0.21
M8	Merlot	6	French	70	6.00 ±0.07
M9	Merlot	10	Macedonian	30	8.98 ±0.23
M10	Merlot	10	Macedonian	70	8.65 ±0.14
M11	Merlot	10	French	30	7.91 ±0.05
M12	Merlot	10	French	70	7.01 ±0.03

[a]Expressed as mmol Trolox per liter. Each value corresponds to the mean and standard deviation of two repetitions ±sd.

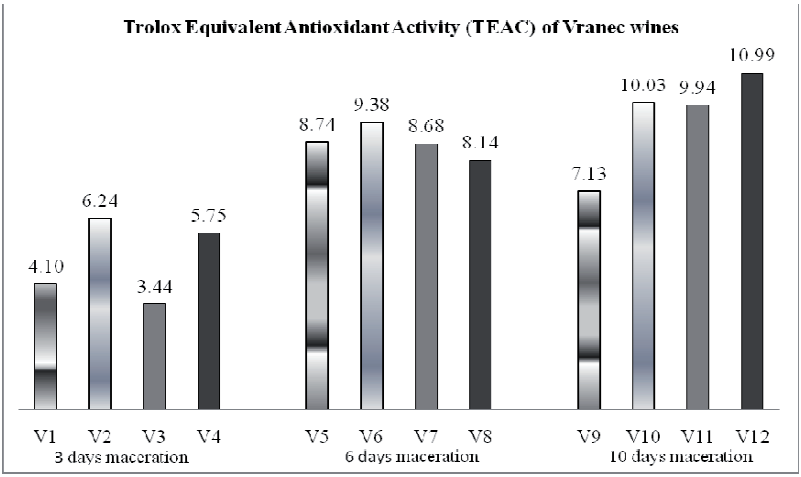


Fig. 36. Antioxidant activity of Vranec wines

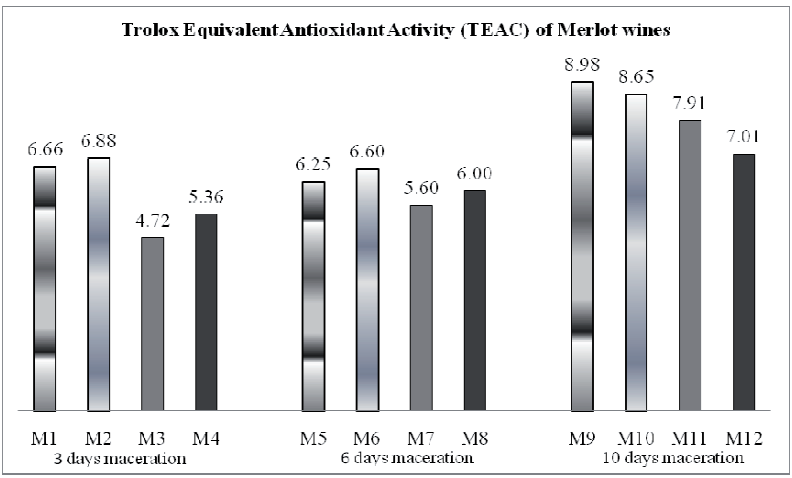


Fig. 37. Antioxidant activity of Merlot wines

Results presented in Table 4 and 5 and Figures 36-37showed strong influence of vinification technology on antioxidant activity of wines.

Results obtained for measurements of Vranec wines were in the range of 3.44 for wine produced with 3 days of maceration time, by application of French yeast and 30 ppm SO_2 and 10.99 for wine produced with 10 days of maceration time, French yeast and 70 ppm SO_2.

Higher values of antioxidant capacity were obtained for wines using 70 ppm instead of 30 ppm SO_2 in all Vranec wines except for wines produced with French yeast and 6 days of maceration time. Vranec wines produced by application of "Vinalco" yeast show higher antioxidant activity in comparison with the corresponding wines from the same grape variety produced by application of "Levuline CHP" yeast. The only exception was Vranec wines with 10 day of maceration time where implication of "Levuline CHP" indicated higher antioxidant activity than "Vinalco" under same vinification procedure.

The antioxidant activity of Merlot wines was in the range from 4.72 for wine produced with 3 days of maceration time, French yeast and 30 ppm SO_2 to 8.98 for wine with 10 days of maceration time, Macedonian yeast and 30 ppm SO_2. Higher values for antioxidant activity were obtained for wines produced with prolonged time of maceration and application of "Vinalco" yeast. The effect of sulfur dioxide did not affect significantly the antioxidant activity of Merlot wines probably because of the lower level of polyphenolics in this grape variety in comparison with Vranec grapes.

3.2.1. Influence of grape variety

The antioxidant activity of Vranec and Merlot wines produced under same vinification procedure were compared. The results are shown in Figures 38-41.

Vranec wine V1, V5 and V9 produced by application of Macedonian yeast "Vinalco" and addition of 30 ppm SO_2 reached highest concentration of total polyphenolics during 6 days of maceration time (Fig. 38 and 39). The results are in agreement with the total phenolic content of the same wines examined from working group of Ivanova (Ivanova *et al*., 2010). Also, for all other Vranec wines, the difference between antioxidant capacity for 6 and 10 days was not statistically significant. On the other hand, wines from Merlot grape variety indicated a maximum for antioxidant activity for 10 days of maceration time.

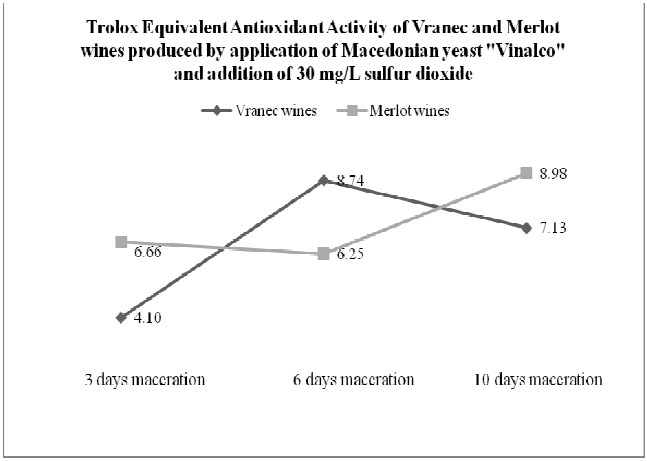


Fig. 38. Comparison between antioxidant activity of Vranec and Merlot wines during vinification by application of Macedonian yeast "Vinalco" and 30 mg/L SO_2

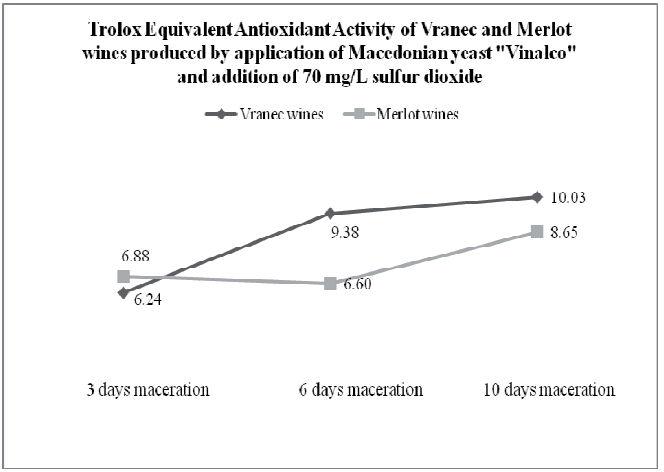


Fig. 39. Comparison between antioxidant activity of Vranec and Merlot wines during vinification by application of Macedonian yeast "Vinalco" and 70 ppm SO_2

Results obtained from investigation of antioxidant capacity for Merlot wines produced with Macedonian yeast and 30 ppm SO_2 indicated strong relationship with the level of total phenolics and total anthocyanins examined by Ivanova *et al.* (Ivanova, Stefova *et al*., 2009). The level of total phenolics for Merlot wine with 6 days measured after maceration (2937 ± 3.56 mg/L) was slightly lower from the same wine produced by 3 days of maceration time (3006 ± 0.76 mg/L) and maximal concentration was reached by 10 days of maceration time (3467 ± 3.08 mg/L) (Ivanova, Stefova *et al*., 2009). The same tendency was noted for total anthocyanins. Concentrations of total flavonoids gradually increased during maceration from 413 ± 3.78 mg/L for 3 days, 539 ± 2.69 mg/L for 6 days and finally reached the maximum concentration of 566 ± 1.34 mg/L for 10 days of maceration time. The concentration of catechins reached their maximum during prolonged skin contact (332 ± 2.29 mg/L for 10 days of maceration)

(Ivanova, Stefova *et al*., 2009). During 3 days of maceration, mainly anthocyanins and tannins from the skins are extracted and this period of contact with the grape mash is not sufficient for extraction of seeds tannins. Generally speaking, from the results presented in Fig. 36-39 Vranec wines had higher antioxidant activity than Merlot wines. The level of total phenols also showed higher values for Vranec than Merlot wines (Ivanova *et al*., 2010, Ivanova *et al*., 2009). It can be concluded that the observed differences of both types of wines during winemaking are due to the different varieties.

3.2.2. Influence of maceration time

Maceration time had the strongest influence on the antioxidant activity of the wines. It is obviously that all wines had lowest antioxidant capacity after 3 days of maceration. The contact between the seeds and skins of the grapes was not long enough for complete extraction due to the lower concentration of ethanol at the beginning of the vinification. Vranec wines showed maximal extraction during 6 days of maceration time. This finding is in agreement with findings of Kovac *et al*. (1992) who showed that maximal extraction of polyphenols was during 6 to 7 days for the same grape variety. Furthermore those wines had better sensory rating. During prolonged maceration the concentration of proanthocyanidins increases and the level of anthocyanins decreases which can be the reason for maximal antioxidant activity during 6 days of maceration time (Mazza *et al*., 1999; Spranger *et al*., 2004; Budić-Leto et *al*., 2008).

Merlot wines showed a maximum for antioxidant activity during prolonged pomace contact. Lower concentration of polyphenolics probably needs

longer period of extraction which resulted in the highest yields of polyphenolics after 10 days of maceration.

Statistical analysis for Vranec and Merlot wines in Table 6 and 7 proved that maceration time has the highest effect on antioxidant activity of the wines (x1 factor is 0,000 for both type of wines).

3.2.3. Influence of yeast

The type of the yeast influenced the antioxidant activity of the wines. It is notable that in wines produced with Macedonian yeast "Vinalco" higher antioxidant activity was observed in comparison with the wines produced with French yeast "Levuline CHP" (Table 4 and 5).

"Vinalco" and "Levuline CHP" belong to the *Saccharomyces cerevisiae* type of yeasts. The "Vinalco" yeast was isolated and selected from the region of Macedonia by Yeast Factory, Bitola, Macedonia. The yeast "Levuline CHP" was isolated from the terroirs of Champagne and selected by CIVC 8130 Interprofessional Committee of Champagne Wines, France.

Morata *et al*. found that different anthocyanins had different affinity to the cell wall of the different strains of *Saccharomyces cerevisiae* yeasts. According to their findings acetyl derivatives (*p*-coumaroyl and acetyl) were better adsorbed than non-acyl derivatives. Also, anthocyanins with a greater degree of methoxylation (malvidin and peonidin) were better adsorbed than the hydroxylated ones (delphinidin and petunidin). It has been concluded that adsorption involves a hydrophobic interaction. According to these findings, the adsorption of peonidin and its derivatives was slightly greater than that of malvidin and its derivatives which was explained with the steric differences of these molecules providing some

adsorption advantage to peonidin (Morata *et al*., 2003). Different *Saccharomyces cerevisiae* strains had the highest influence of anthocyanins and hydroxycinnamic acid in red Tempranillo wines while non-anthocyanins such as hydroxybenzoic acid and flavanols were less affected (Monagas *et al*., 2007). During the first week of vinification only few monomeric polyphenols were absorbed on the yeast walls. The low and high polymeric tannins were not absorbed and remained into the wine. Only polar condensed tannins have higher affinity of adsorption on yeast walls (Mazauric & Salmon, 2005).

It can be assumed that Macedonian and French yeast strains possess with different affinities to anthocyanins. This probably indicates a lower adsorption of these classes of phenolics on the walls of Macedonian yeast "Vinalco". This may delay the polymerization process in comparison with French yeast "Levuline CHP" (Caridi *et al*., 2004). The other reason for higher antioxidant activity of wines produced with "Vinalco" can be due to different kinetics of sugar conversion into ethanol of the two applied yeasts strains. Possibility of faster fermentation leads to higher percentage of alcohol and better extraction of polyphenols from skin and seeds of the grapes.

The effect of yeast was proven by statistical analysis in Table 6 and 7 (factor x2 for Vranec wines 0.015 and for Merlot wines 0.000)

3.2.4. Influence of SO_2

The antioxidant and antimicrobial activity of SO_2 is well-known and protects the wine from oxidation reactions. Its reaction with hydrogen

peroxide and acetaldehyde also inhibits the polymerization of anthocyanins (Garrido *et al*., 2011).

Effect of SO_2 was statistically significant only for Vranec wines because those wines were richer in polyphenols than Merlot wines (factor x3 is 0.000). All Vranec wines, produced with the same vinification procedure showed higher antioxidant capacity by application of 70 mg/L SO_2. If we compare the difference between values of antioxidant activity presented on Fig. 40 and 41 we can conclude that higher dosage of sulfur dioxide improves the extraction of polyphenolics in Vranec wines from 3 and 10 days of maceration. Higher level of SO_2 did not have significant effect on Merlot wines probably due to their lower level of polyphenolics. The results leaded to the conclusion that SO_2 as antioxidant did not contribute significantly to the total antioxidant activity of red wines because of high concentrations of polyphenolics (Maereschi *et al*., 1992). Those findings are in agreement with previous published results which explain interactions of sulfur dioxide with polyphenols in wines and its role as regulator in formation of polymeric pigments and changes in tannins structures and its ability to reduce oxidized polyphenols to their reduced form (Danilewicz *et al*., 2008; Tao *et al*., 2007).

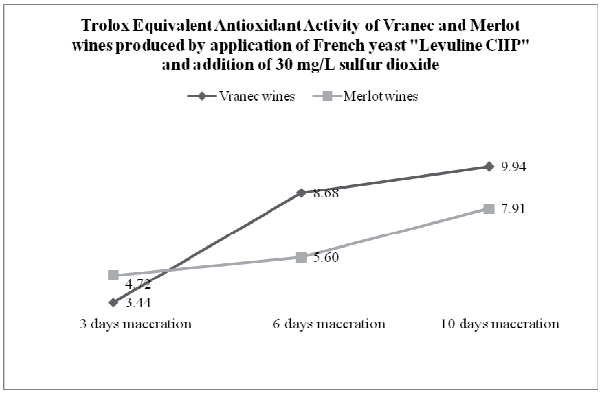


Fig. 40. Comparison between antioxidant activity of Vranec and Merlot wines during vinification by application of French yeast "Levuline CHP" and 30 mg/L SO_2

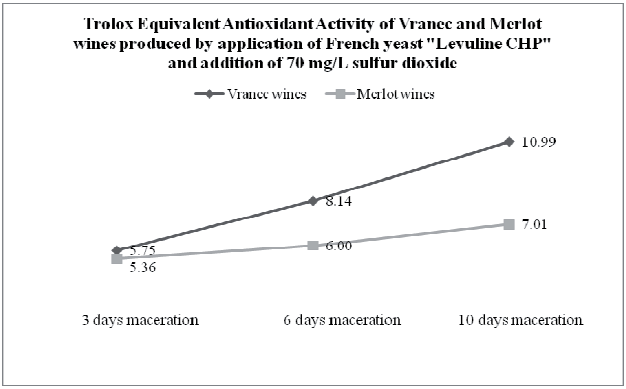


Fig. 41. Comparison between antioxidant activity of Vranec and Merlot wine during vinification with French yeast "Levuline CHP" and 70 mg/L SO_2

Using the General Linear Model (GLM) is possible to calculate the most significant factor and less significant factor from vinification which contribute to the antioxidant capacity of the wines. In Table 6 and 7 results are presented from three factors in wine-making technology (maceration

time as x1, type of yeast as x2 and concentration of SO_2 as x3) and interaction effects between those three factors.

Table 6. Models for the General Linear Model (GLM) for Vranec wines

Response variable		Main effects			Interaction effects		
		x_1	x_2	x_3	$x_1 x_2$	$x_1 x_3$	$x_2 x_3$
Antioxidant activity	*p*-value	**0.000***	**0.015***	**0.000***	**0.004***	**0.000***	0.354
	F-ratio	**298.34**	**7.75**	**143.63**	**8.23**	**32.04**	0.09

x_i: the estimated regression coefficient for the main effects

$x_i x_j$: the estimated regression coefficient for the interaction effects

1: Time of maceration; 2: Type of yeast; 3: Concentration of sulfur dioxide

*: significant ($p < 0.05$)

Table 7. Models for the General Linear Model (GLM) for Merlot wines

Response variable		Main effects			Interaction effects		
		x_1	x_2	x_3	$x_1 x_2$	$x_1 x_3$	$x_2 x_3$
Antioxidant activity	*p*-value	**0.000***	**0.000***	0.123*	**0.001***	**0.000***	0.476
	F-ratio	**262.86**	**149.65**	2.70	**12.39**	**18.04**	0.54

x_i: the estimated regression coefficient for the main effects

$x_i x_j$: the estimated regression coefficient for the interaction effects

1: Time of maceration; 2: Type of yeast; 3: Concentration of sulfur dioxide

*: significant ($p < 0.05$)

Generally concluded and confirmed by statistical analysis, the time of maceration and type of yeast are the most significant factors. The concentration of SO_2 was not significant factor for antioxidant activity of Merlot wines.

In terms of interaction effect of independent variables, the independent interaction between maceration time and type of yeasts (x1x2) and maceration time and concentration of SO_2 (x1x3) had significant effect on the response variable which indicate that effects are not independent and many interactions in the complex matrix of wine are possible.

3.2.5. Influence of wine-making on the sensory analysis of wines

The sensory analysis of the wines obtained under different wine-making technologies using 3, 6 and 10 days of maceration time, two types of yeasts and two doses of SO_2 was performed by evaluating the overall organoleptic quality. The scores for the wines are presented in Fig. 42. PCA score plots were used to determine whether 24 wines could be grouped into different classes. To focus on the differences among the wines obtained by different winemaking technologies and overall sensory quality, cluster observation and cluster variable dendrograms were constructed using the nearest neighbour (Fig. 42). The results indicated that first two principal components explained 85.06% and 7.5% of the total variability, respectively. Except for the Merlot wine M7, PCA plot showed clear classification of the wines into two groups located in PC1. On the positive side of PC1 is the group of Vranec and Merlot wines produced by 3 days of maceration time. On the negative part of PC1 is the group of wines produced with 6 and 10 days of maceration time.

3. Results and Discussion

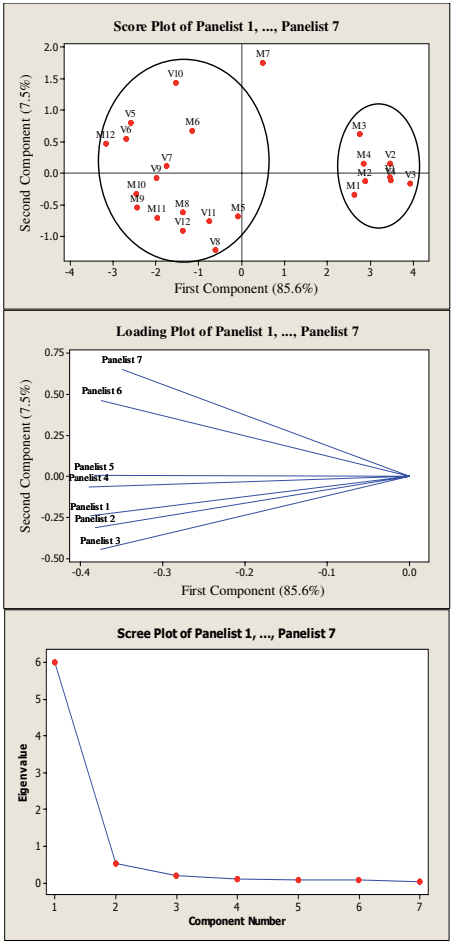


Fig. 42. PCA scatter plots and cluster dendrograms discriminating the overall sensory quality of wines obtained under different winemaking technologies into different classes.

The PCA analysis is in agreement with GLM model due to indication that maceration time is the most important factor during vinification. The behavior can be due to the highest concentration of phenolic compound formed after 6 days for Vranec and after 10 days of maceration time for

Merlot wines. Those compounds were responsible for the favorable taste of the wines. The findings were in agreement with previously published literature that prolonged maceration time enables a better extraction of tannins, the latter being responsible for astringency of the wine (Torrens *et al*., 2008; Domizio *et al*., 2007). Most of the panelists decided that wines produced with Macedonian yeast "Vinalco" were richer in fruity notes and had a higher astringency than the corresponding wines produced with French yeast "Levuline CHP". Different yeasts are known to influence considerably the volatile composition of a wine (Callejon *et al*., 2010).

In conclusion, the best wine-making procedure for Vranec wines was observed with 6 days of maceration time, application of Macedonian yeast "Vinalco" and addition of 70 mg/L SO_2. The best taste of Merlot wines was observed using prolonged maceration time of 10 days, application of Macedonian yeast "Vinalco" and 70 ppm SO_2.

3.3. Isolation of anthocyanins by high-speed countercurrent chromatography and application of the colour activity concept to different varieties of red grape pomace from Macedonia

The third chapter of this dissertation describes the isolation of anthocyanins-3-*O*-glycosides from three varieties of grape pomace "Pinot Noir", "Merlot" and "Vranec" by high speed countercurrent chromatography (HSCCC) and application of colour activity concept for distinguishing the particular contribution of the most abundant anthocyanins-3-*O*-glycosides to the colour of the different varieties of grape pomace.

The samples of three varieties of grape pomace obtained after 20 days of maceration were washed with Nanopure® water, lyophilized and crushed into a powder. After removing of fatty acids with hexane, the anthocyanins from crude samples were extracted with methanol acidified by formic acid in ratio 19:1. In order to remove sugars, salts and other impurities from crude anthocyanin extracts, solid phase extraction by using XAD-7 resin was applied.

The procedure for obtaining the crude anthocyanin extracts is schematically presented in Fig. 43.

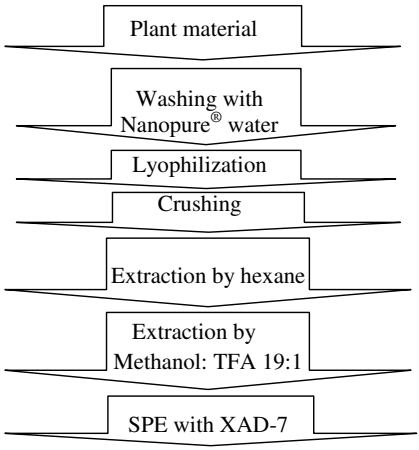


Fig. 43. Schematic diagram for the extraction of anthocyanins from grape pomace

The extract obtained from "Vranec" grape pomace had the darkest violet-blue color. The extract from "Merlot" variety had red-violet color and "Pinot Noir" grape pomace had dark red color. The yield of anthocyanin-enriched extract from three consecutive extractions of grape pomace from "Vranec" variety was 3.7 g, from "Merlot" variety 3.1 g and "Pinot noir" variety 1.5 g per 500 g of dry powdered grape pomace.

The pigments from purified extracts were separated by high speed triple coil countercurrent chromatography. The solvent system consisted of MTBE/*n*-butanol/acetonitrile/water 2:2:1:5 *v/v/v/v* acidified with 0.1% trifluoracetic acid. This combination of polar solvents acidified with trifluoracetic acid enables formation of purple red colored flavylium ion which has maximal absorbance at 520 nm and support separation of pigments trough formation of an ion pair. 1.0 g of anthocyanin-enriched

extracts from each variety of grape pomace was injected into the HSCCC system.

The HSCCC chromatograms of the anthocyanin-enriched grape pomace extract of “Pinot noir”, “Merlot” and “Vranec” are shown in Fig. 44-46, respectively. If we compare the three CCC chromatograms we can conclude that the quantity of isolated pigments is the lowest in “Pinot Noir” grape pomace and the largest abundance of isolated anthocyanins-3-*O*-glycosides is obtained from the sample of “Vranec” grape variety. The amounts of crude anthocyanin extract (obtained by application of solid phase extraction with XAD-7 resin) per one kilogram of grape pomace are presented in Table 8.

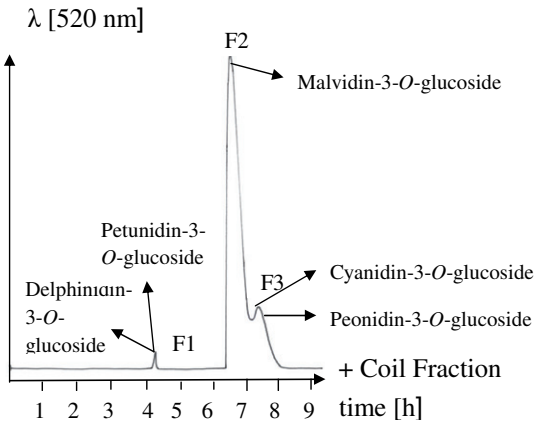


Fig. 44. HSCCC separation of anthocyanins from “Pinot noir” grape pomace at 520 nm

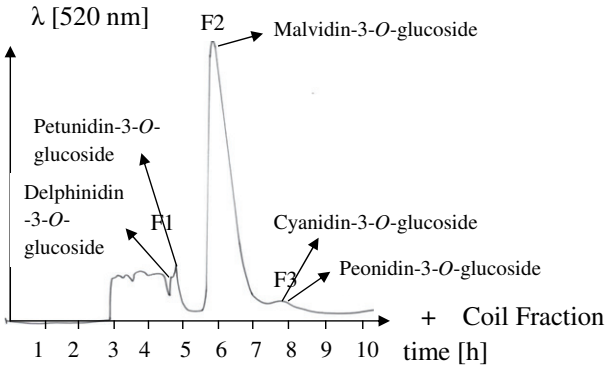


Fig. 45. HSCCC separation of anthocyanins from "Merlot" grape pomace at 520 nm

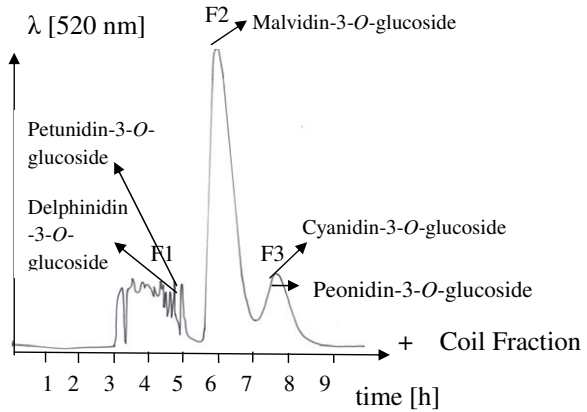


Fig. 46. HSCCC separation of anthocyanins from "Vranec" grape pomace at 520 nm

Table 8. Amount of crude anthocyanin extract from solid phase extraction with XAD-7 resin from "Pinot Noir", "Merlot" and "Vranec" grape pomace

Variety of grape pomace (1 kg)	*Crude anthocyanin XAD-7 extract (g)*
Pinot Noir	3
Merlot	6,2
Vranec	7,4

For HPLC separation of "CCC" fractions a binary gradient of a mixture of water/acetonitrile/formic acid was used. Linear gradient was as follows: A 83/7/10; B: 40/50/10 (v/v/v); % A: 0 min. 94%, 20 min. 80%, 35 min. 60%, 35 min. 40%, 40 min. 90%, 45 min and 55 min 94%. Flow rate was set at 0.5 mL/min.

Fraction F1 was a mixture of dephinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside. Fig. 47 represents total ion chromatogram (TIC) and pseudomolecular ions and fragments of delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside from F1 fraction of "Merlot" grape pomace before purification. For separation of this anthocyanin mixture preparative HPLC was used. The purity of isolated pigments was determined by the "DAD" chomatograms presented in Fig. 48 and 52 for delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside, respectively. After reaching the purity of 95.5 % for delphinidin-3-*O*-glucoside, the NMR structure elucidation was performed in order to confirm the tentative structure obtained from HPLC-MS/MS. Fig. 50 and 51 present ^{1}H NMR and ^{13}C NMR spectra of the isolated pigment. The purity of isolated petunidin-3-*O*-glucoside was 97.2

% and its proton and carbon NMR spectra are presented in Fig. 53 and 54, respectively.

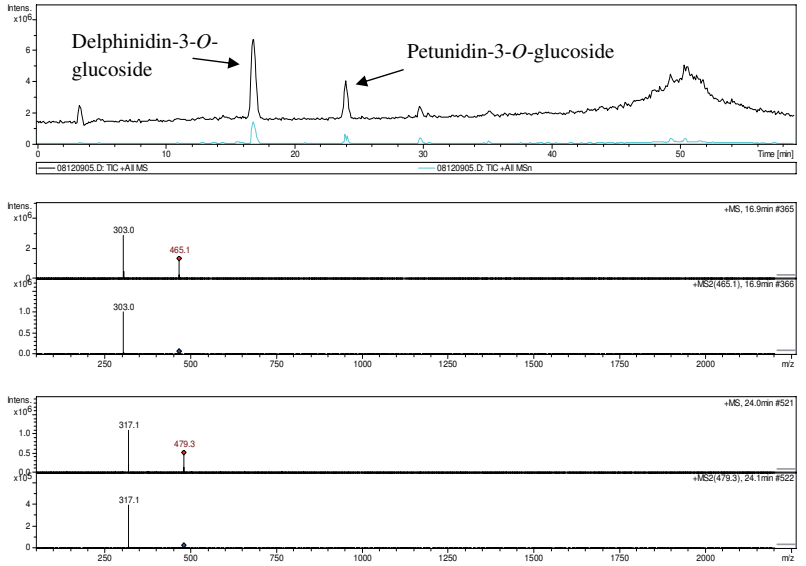


Fig. 47. Total ion chromatogram (TIC) and pseudomolecular ions and fragments of delphinidin-3-*O*-glucoside and petunidin-3-*O*-glucoside from F1 fraction of "Merlot" grape pomace before purification

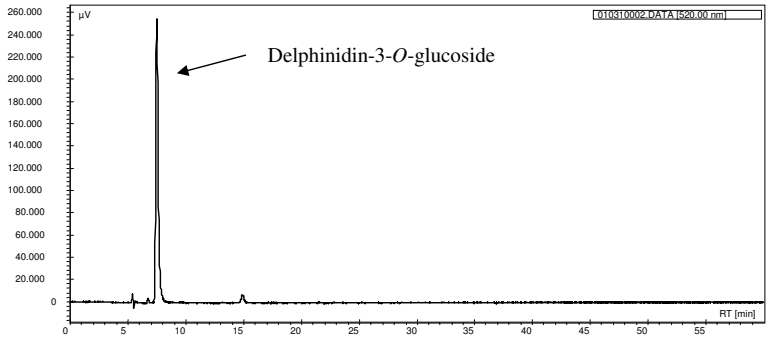


Fig. 48. HPLC-DAD chromatogram of delphinidin-3-*O*-glucoside (purity 95.5%)

3. Results and Discussion

Signals of NMR spectra were assigned according to the structure of anthocyanin-3-*O*-glucoside presented in Fig. 49 and Tab. 9.

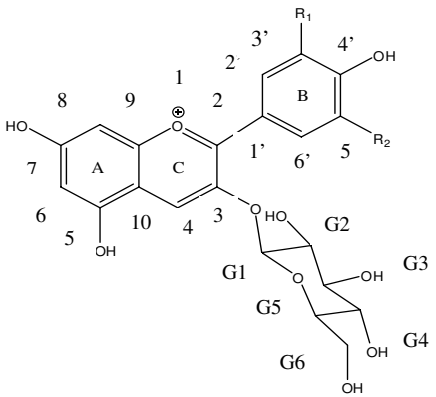


Fig. 49. Chemical structure of anthocyanin-3-*O*-glucoside

Table. 9. Structure of anthocyanin-3-*O*-glucoside

	Delphinidin -3- glucoside	***Cyanidin- 3- glucoside***	***Petunidin -3- glucoside***	***Peonidin- 3- glucoside***	***Malvidin- 3- glucoside***	***Malvidin-3-p-coumaroyl-glucoside***
R1	-OH	-OH	$-OCH_3$	$-OCH_3$	$-OCH_3$	$-OCH_3$
R2	-OH	-H	-OH	-H	$-OCH_3$	$-OCH_3$

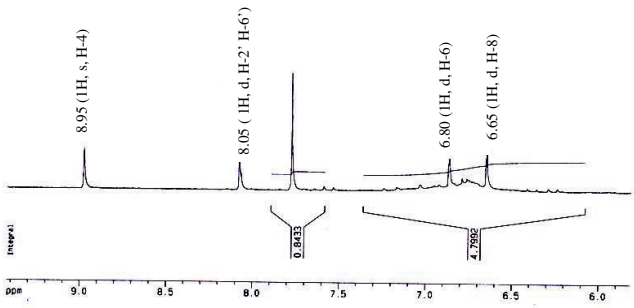


Fig. 50. ^{1}H NMR spectroscopic data of delphinidin-3-*O*-*β*-*D*-glucopyranoside (Delphinidin-3-*O*-glucoside)

Fig. 50. presents the part of ^{1}H-NMR spectra of the isolated delphinidin-3-*O*-β-D-glucopyranoside. The chemical shifts obtained from ^{1}HNMR confirmed the identity of pigment. Singlets from the aglycone moiety at δ 8.95 (1H, s, H-4), δ 8.05 (1H, d, H-2' H-6'), δ 6.67 (1H, s, H-8), δ 6.80 (1H, d, H-6), doublet and multiplets from the glucopyranoside moiety δ 5.30 (1H, d, G-1), δ 3.95 (1H, m, G-6), δ 3.70 (1H, d, G-6), δ 3,65 (4H, m, G-2, G-3, G-4, G-5) were in good accordance with the results from the ^{1}H NMR spectra of delphinidin-3-glucoside in the work of Bjorøy *et al.* (2007), Ha *et al.* (2010), Nickavar & Amin (2004), Takeoka *et al.* (1997) and Kuskoski *et al.* (2003).

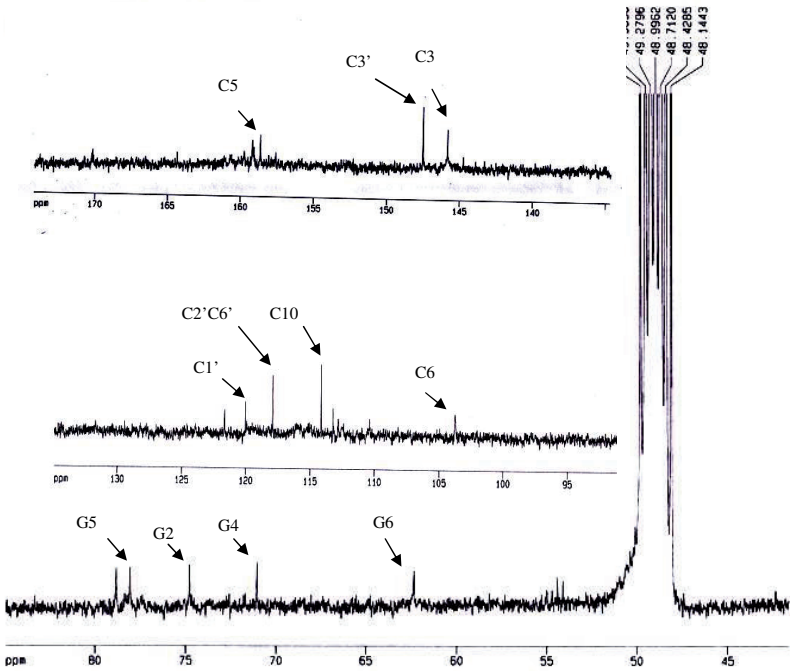


Fig. 51. ^{13}C NMR spectroscopic data of delphinidin-3-*O*-β-D-glucopyranoside (delphinidin-3-*O*-glucoside)

Fig. 51 presents the ^{13}C NMR spectra of the isolated delphinidin-3-*O*-β-D-glucopyranoside. The chemical shifts obtained from ^{13}C NMR of delphinidin-3-glucoside gave further confirmation of the structure of the isolated pigment. Signals from the aglycone moiety δ 158.68 (C5), δ 147.55 (C3'), δ 145.86 (C3), δ 120.05 (C1'), δ 117.93 (C2', C6'), δ 113.23 (C10) and from the glucopyranoside moiety δ 78.84 (G5), δ 74.79 (G2), δ 71.08 (G4) and δ 62.36 (G6) were in good agreement with the published results from ^{13}C NMR spectra of delphinidin-3-*O*-glucoside in the work of Bjorøy *et al*., (2007), Ha *et al*. (2010) and Tsuda *et al*. (1994).

3. Results and Discussion

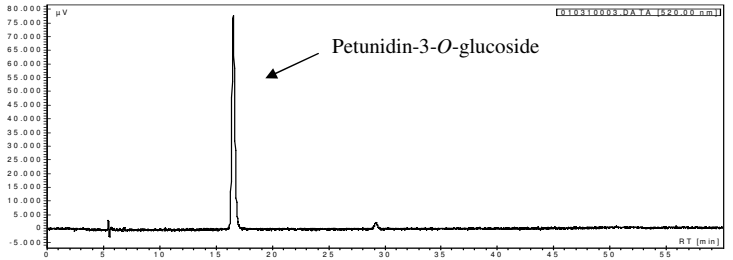


Fig. 52. HPLC-DAD chromatogram of petunidin-3-*O*-glucoside (purity 97.2%)

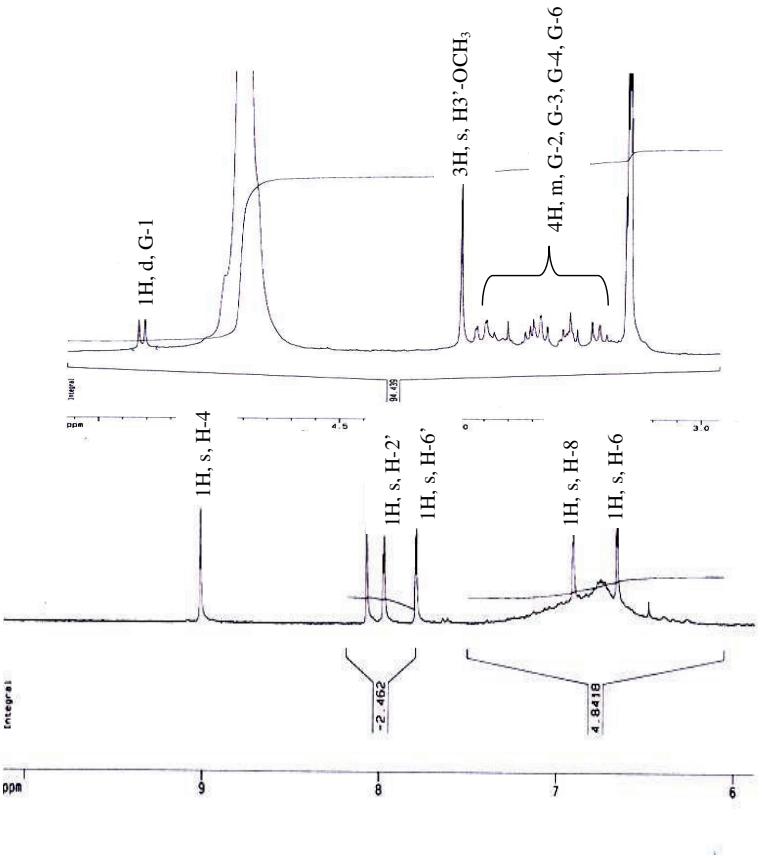


Fig. 53. ^{1}H NMR spectroscopic data of petunidin-3-*O*-β-D-glucopyranoside (petunidin-3-*O*-glucoside)

Fig. 53 presents the ^{1}H NMR spectra of the isolated petunidin-3-O-glucoside. The chemical shifts obtained from the ^{1}H NMR of petunidin-3-glucoside confirmed the identity of the pigment. Singlets from aglycone moiety δ 9.0 (1H, s, H-4), δ 4.00 (3H, s, H3-OCH_3) doublets from aglycone moiety δ 7.95 (1H, d, H-2'), δ 7.80 (1H, d, H-6'), doublets from glucopyranoside moiety δ 5.30 (1H, d, G-1) and multiplets from glucopyranoside moiety δ 3.90-3.40 (4H, m, G-2, G-3, G-4, G-6) were in good accordance with the results from the ^{1}H NMR spectra of petunidin-3-glucoside in the work of Nickavar & Amin (2004) and Kuskoski *et al.* (2003).

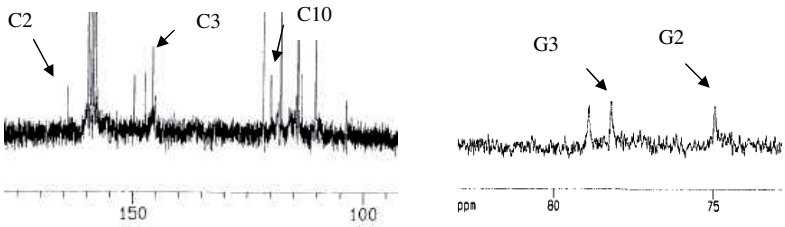


Fig. 54. ^{13}C NMR spectroscopic data of petunidin-3-*O*-*β*-*D*-glucopyranoside (petunidin-3-*O*-glucoside)

Fig. 54 presents the part of ^{13}C NMR spectra of the isolated petunidin-3-*O*-*β*-*D*-glucopyranoside. Shifts from δ 164.25 (C2), δ 158.72 (C5), δ 149.85 (C3'), δ 147.51 (C4'), δ 145.8 (C5'), δ 145.80 (C4'), δ 113.49 (C6'), δ 78.9 (G3), δ 78.2 (G5), δ 74.96 (G2), δ 71.19 (G1), δ 62.42 (G6) confirmed the structure of the isolated anthocyanin. The signals from ^{13}C NMR of the pigment were in correspondance to the signals for petunidin-3-*O*-glucoside isolated from *Liriope platyphylla* fruits in the work of (Lee & Choung, 2011).

The second fraction F2 was the main fraction from the "CCC" chromatograms of the three varieties of grape pomace. It contained the highest amount of isolated pigment with maximal abundance of 19 mg/g of crude extract in "Vranec" grape pomace.

Fig. 55 presents the total ion chromatogram (TIC) and mass spectrometric data of malvidin-3-glucoside from F2 fraction of "Vranec" grape pomace before purification. After purification of the isolated anthocyanin (purity of 96.8 %) NMR stucture elucidation was performed. In Fig. 57 and 58 the ^{1}H NMR and ^{13}C NMR spectra of isolated malvidin-3-glucoside are presented.

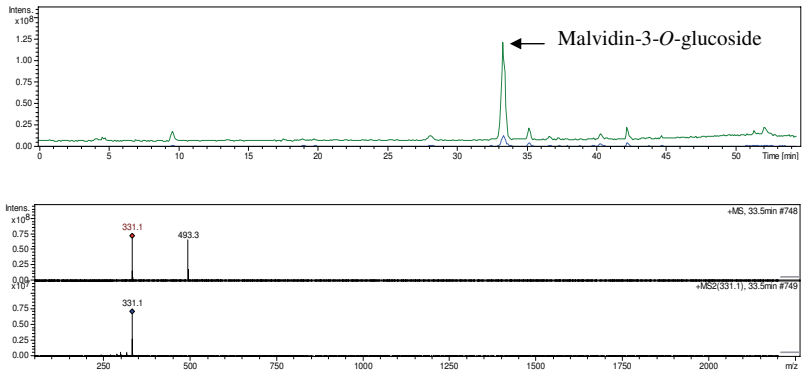


Fig. 55. Total ion chromatogram (TIC) and mass spectrometric data of malvidin-3-glucoside from F2 fraction of "Vranec" grape pomace before purification

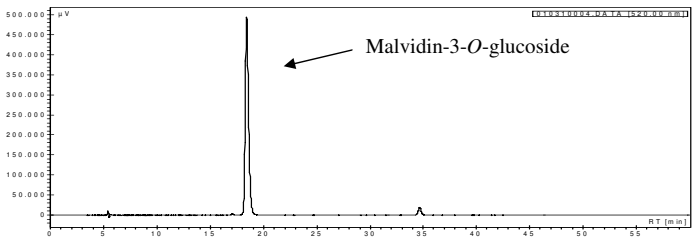


Fig. 56. HPLC-DAD chromatogram of malvidin-3-*O*-glucoside (purity 96.8%)

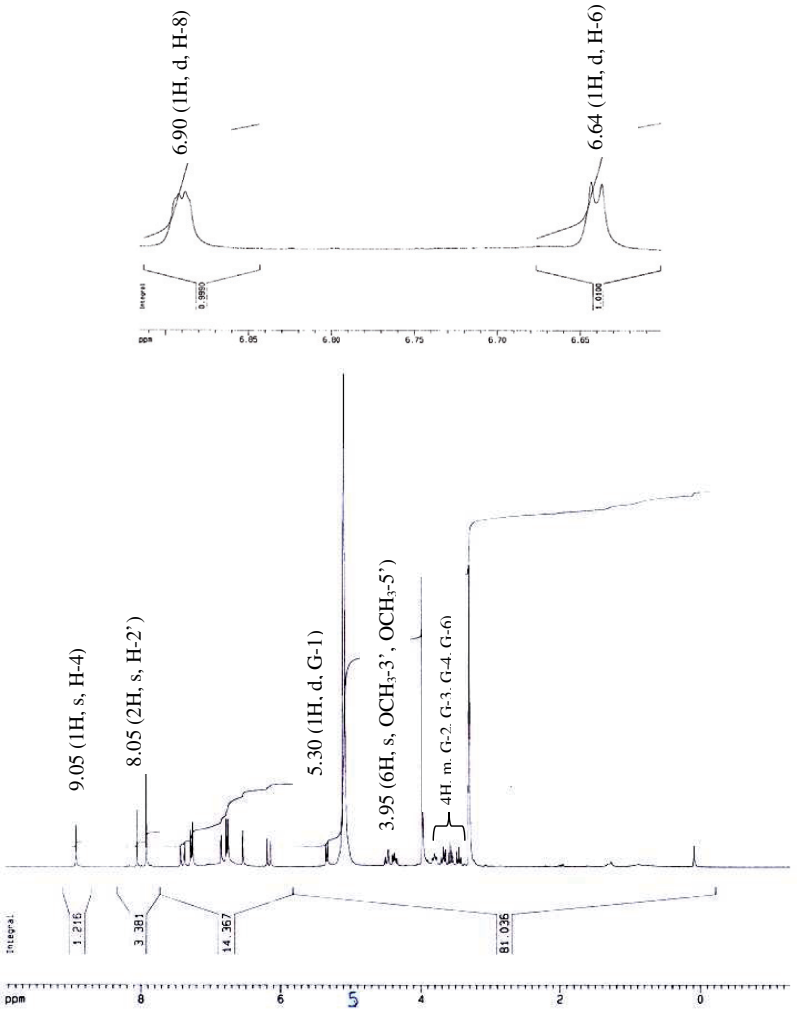


Fig. 57. ^{1}H NMR data of malvidin-3-*O*-*β*-*D*-glucopyranoside (malvidin-3-*O*-glucoside)

In Fig. 57 the ^{1}H NMR spectra of the isolated malvidin-3-O-glucoside is depicted. The chemical shifts obtained from ^{1}H NMR of the most abundant pigment in grape pomace confirmed its identity. Singlets from the aglycone

moiety δ 9.05 (1H, s, H-4), δ 8.05 (2H, s, H-2'), δ 3.95 (6H, s, OCH_3-3', OCH_3-5'), doublets from the aglycone moiety δ 6.90 (1H, d, H-8), δ 6.64 (1H, d, H-6) and multiplets from the glucopyranoside moiety δ 5.30 (1H, d, G1) and δ 3.45-3.90 (4H, m, G2, G3, G4, G5) were in good accordance with the results from the ^{1}H NMR spectra of malvidin-3-glucoside reported in the work of Takeoka *et al*. (1997), Nickavar & Amin (2004), Andersen *et al*. (1995), Atanasova *et al*. (2002), Aguirre *et al*. (2010) and Kuskoski *et al*. (2003).

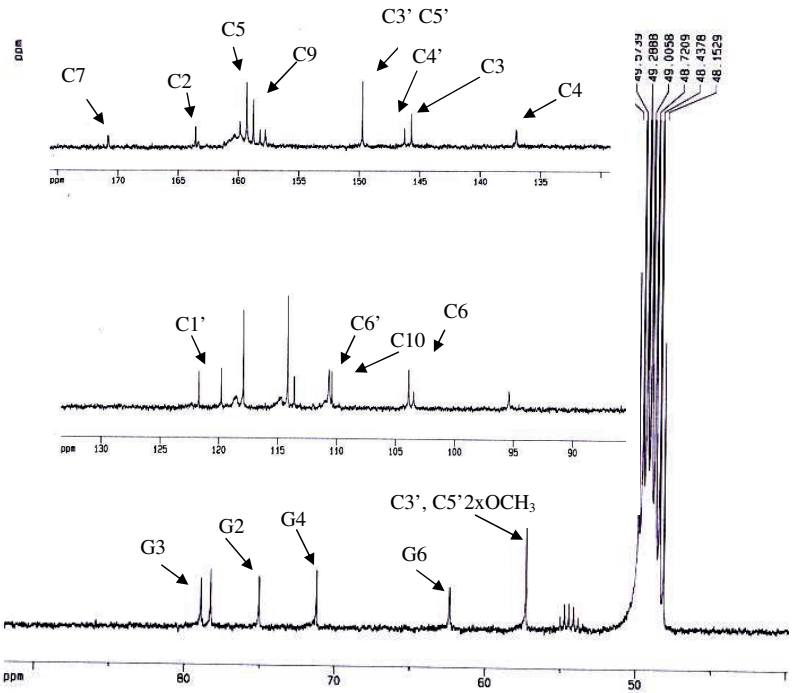


Fig. 58. ^{13}C NMR spectroscopic data of malvidin-3-*O*-*β*-*D*-glucopyranoside (malvidin-3-*O*-glucoside)

Fig. 58 shows the ^{13}C NMR spectra of the same anthocyanin from grape pomace. The chemical shifts obtained from ^{13}C NMR of the most abundant pigment confirmed its identity. Shifts δ 170.10 (C7), δ 163.56 (C2), δ 159.32 (C5), δ 157.79 (C9), δ 149.75 (C3' C5'), δ 146.28 (C-4'), δ 145.69 (C3), δ 137.03 (C4), δ 119.79 (C1'), δ 113.61 (C10), δ 110.40 (C6') δ 103.92 (G1), δ 103.49 (C6) δ 78.28 (G3), δ 75.05 (G2), δ 71.20 (G4), δ 62.39 (G6), δ 57.32 (C3'C5'2xOCH_3) were in good accordance with the results reported for malvidin-3-glucoside by Andersen *et al*. (1995), Atanasova *et al*. (2002) and Aguirre *et al*. (2010).

Furthermore, in very small quantity the anthocyanins cyanidin-3-glucoside and peonidin-3-glucoside were isolated from fraction F3. In Fig. 59 the total ion chromatogram (TIC) and mass spectrometric data of cyanidin-3-*O*-glucoside is depicted.

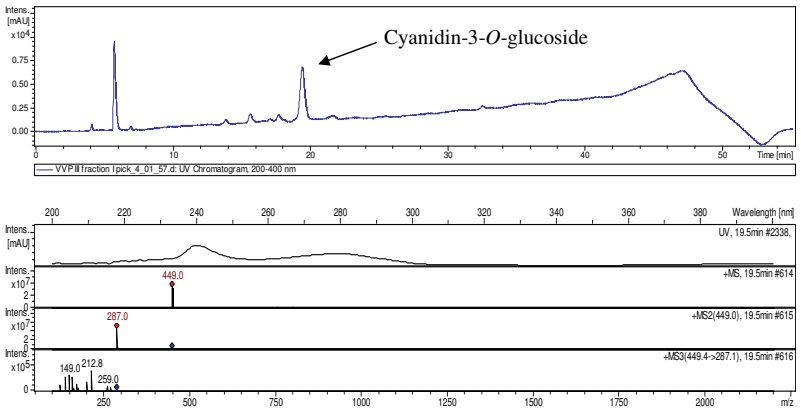


Fig. 59. Total ion chromatogram (TIC) and pseudomolecular ions and fragments of cyanidin-3-*O*-glucoside from F3 fraction of "Merlot" grape pomace before purification

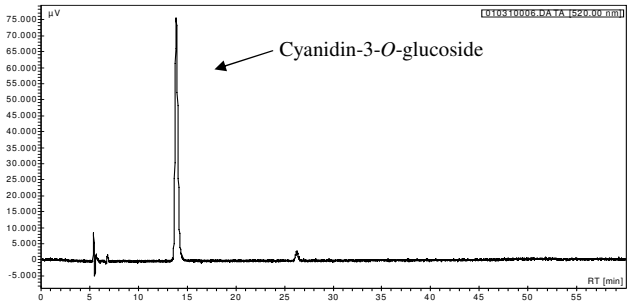


Fig. 60. HPLC-DAD chromatogram of cyanidin-3-*O*-glucoside (98.3%)

After purification of the isolated pigment NMR structure elucidation was performed. The ^{1}H NMR spectra of the compound is not presented since it was reported in the literature and due to the similarity with the other isolated pigments. In the text below the signals obtained from ^{1}H NMR are discussed.

Singlets from aglycone moiety δ 9.0 (1H, s, H-4), doublets from the aglycone moiety δ 8.25 (1H, dd, H-6'), δ 8.05 (1H, d, H-2'), δ 7.05 (1H, d, H-5'), δ 6.70 (1H, d, H-6) and multiplets from the glucopyranoside moiety δ 3.9 (1H, m, G-6), δ 3.55 (2H, m, G-3, G-5), were in good accordance with the results for cyanidin-3-*O*-glucoside by Jordheim *et al*. (2007), Ha *et al*. (2010), Tsuda *et al*. (1994), Yawadio *et al*. (2007) and Kuskoski *et al*. (2003).

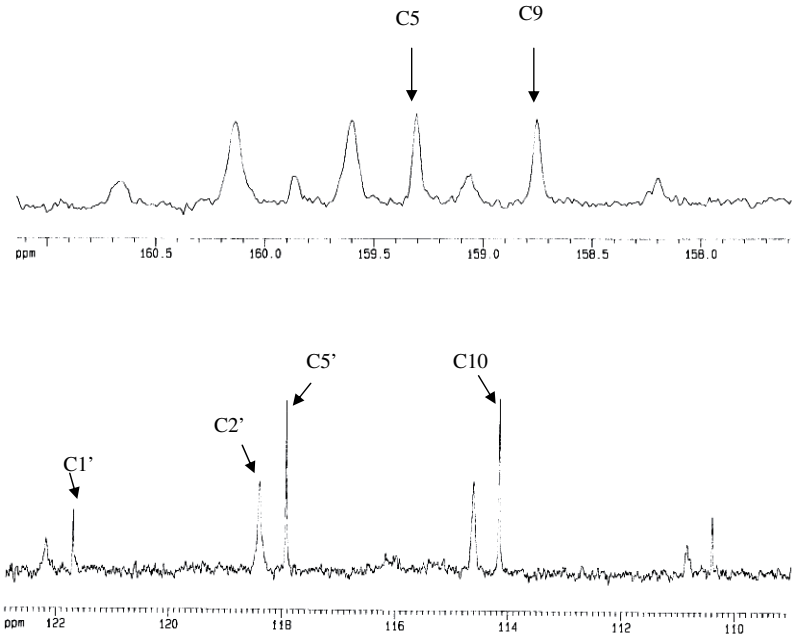


Fig. 61. ^{13}C NMR spectroscopic data of cyanidin-3-*O*-β-D-glucopyranoside (cyanidin-3-*O*-glucoside)

Fig. 61 presents part of the ^{13}C NMR spectra of the same anthocyanin. The chemical shifts obtained from ^{13}C NMR of cyanidin-3-glucoside confirmed the tentative structure from MS/MS fragmentation. Shifts from the aglycon moiety δ 159.60 (C9), δ 159.30 (C5), δ 121.70 (C1'), δ 118.40 (C2'), δ 117.93 (C5') and δ 114.16 (C10) were in good accordance with the results from the ^{13}C NMR spectra of cyanidin-3-*O*-glucoside published by Jordheim *et al*. (2007), Ha *et al*. (2010), Tsuda *et al*. (1994), Yawadio *et al*. (2007) and Kuskoski (2003).

3. Results and Discussion

The total ion chromatogram (TIC), the fragmention and DAD chromatogram of the second isolated pigment from fraction F3 peonidin-3-glucoside (purity 97.4%) is depicted in Fig. 62 and 63.

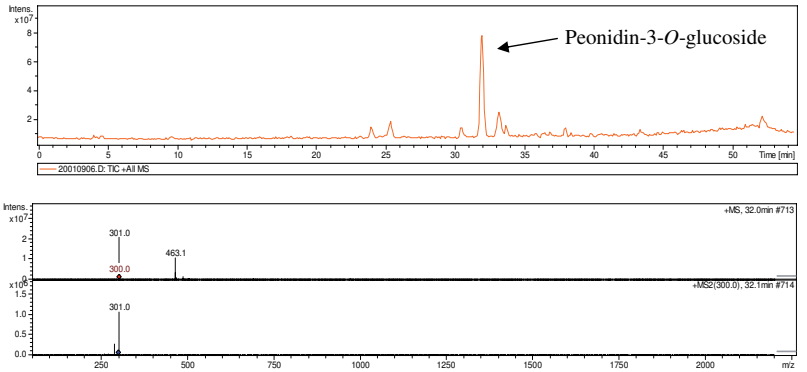


Fig. 62. Total ion chromatogram (TIC) and mass spectrometric data of peonidin-3-glucoside from F3 fraction of "Vranec" grape pomace before purification

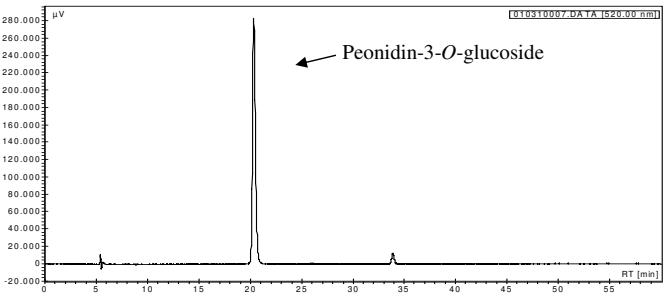


Fig. 63. HPLC-DAD chromatogram of peonidin-3-*O*-glucoside (purity 97.4 %)

The chemical shifts obtained from the ^{1}H NMR spectra of peonidin-3-*O*-glucoside confirmed the tentative structure from HPLC/MS^{n} analysis. Singlets from the aglycone moiety δ 9.00 (1H, s, H-4), δ 8.05 (1H, s, H-4), δ 7.05 (1H, d, H-5'), δ 6.95 (1H, d, H-8), δ 6,70 (1H, s, H-6), doublets from

the glucopyranoside moiety δ 8.3 (1H, dd, H-6'), δ 5.35 (1H, d, G-1), and multiplets from the glucopyranoside moiety δ 3.40-3,75 (4H, m, G-2, G-3, G-4, G-5) were in good accordance with the results obtained by Yawadio *et al*. (2007). Since very small quantity of the pigment was isolated the ^{13}C NMR spectra was not recorded.

The coil fraction of the "CCC" separation was enriched with malvidin-3-*p*-coumaroyl-*O*-*β*-*D*-glucopyranoside. After purification by using preparative HPLC the isolated pigment reached a purity of 95.5 %. The HPLC/MS and DAD chromatograms of malvidin-3-*p*-coumaroyl glucoside are depicted in Fig. 64 and 65, respectively.

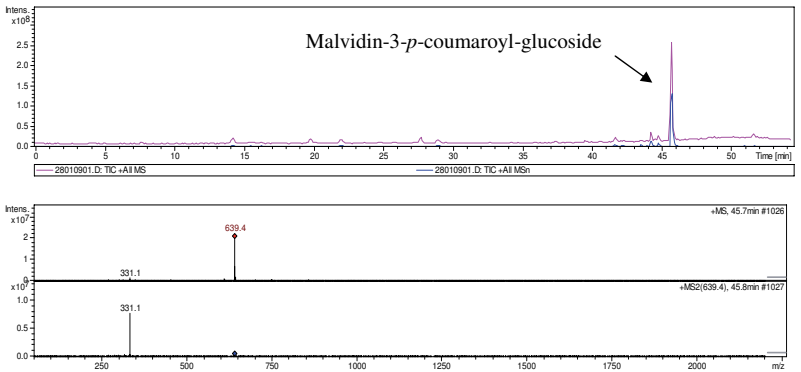


Fig. 64. Total ion chromatogram (TIC) and mass spectrometric data of malvidin-*p*-coumaroyl-glucoside in "Vranec" coil residue before purification

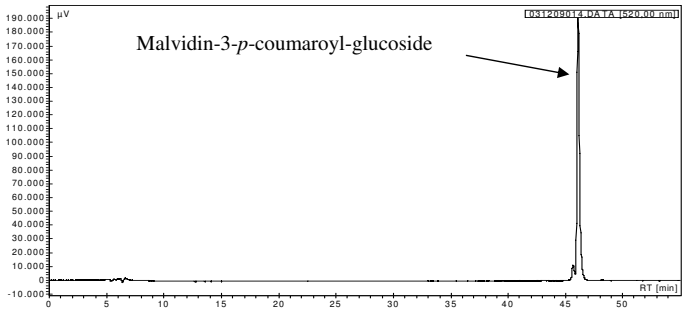


Fig. 65. HPLC-DAD chromatogram of malvidin-3-*p*-coumaroyl-glucoside (purity 95.5 %)

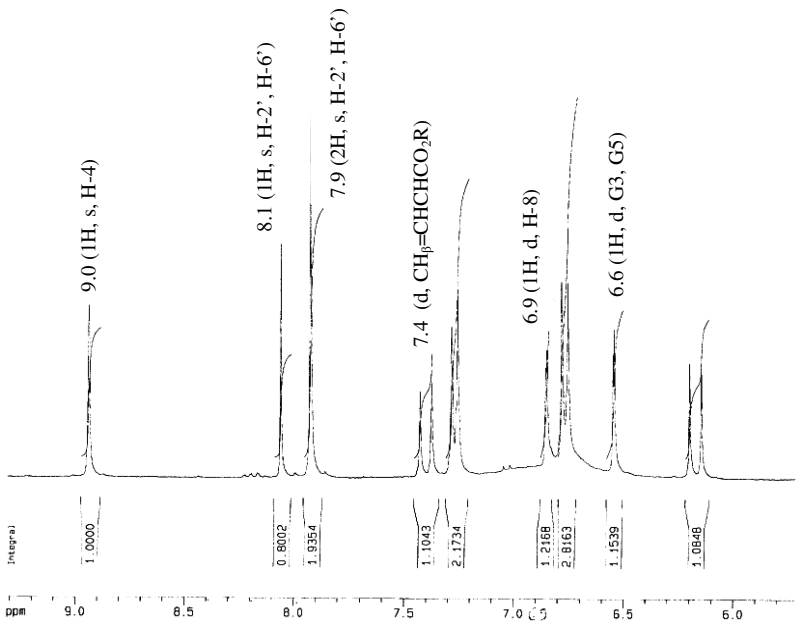
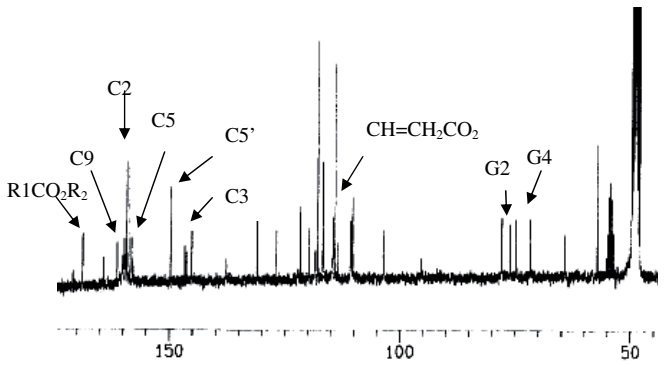


Fig. 66.[1]H NMR spectra of malvidin-3-*p*-coumaroyl-glucoside

Fig. 66 presents the part of [1]H NMR spectra of the isolated malvidin-3-*p*-coumaroyl-glucoside. The chemical shifts obtained from the [1]H NMR

spectra of the second most abundant pigment in grape pomace confirmed its identity. Singlets from δ 9.0 (1H, s, H-4), δ 8.1 (1H, s, H-2', H-6'), δ 7.9 (2H, s, H-2', H-6'), δ 7.4 (d, CH_{β}=$CHCO_2R$), δ 6.9 (1H, d, H-8), δ 7.25 (d, G-2, G-6), δ 6.6 (1H, d, G3, G5), δ 5.4 (1H, d, G1), δ 3.7-3.3 (H5, m, G2, G3, G4, G5, G6) were in accordance with the results from the work of Takeoka *et al*. (1997), Nickavar & Amin (2004), Andersen *et al*. (1995), Atanasova *et al*. (2002), Aguirre *et al*. (2010) and Kuskoski *et al*. (2003). The distance between the signals presented in Fig. 66 was 15 ppm which confirmed that the double bond from the coumaroyl group is "*trans*" configurated.



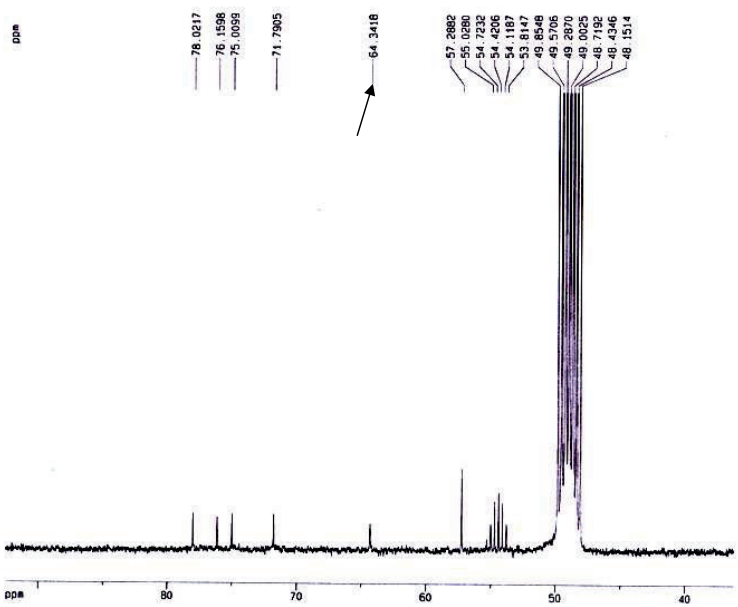


Fig. 67. ^{13}C NMR spectroscopic data of malvidin-3-*p*-coumaroyl-glucoside

Fig. 67 presents the ^{13}C NMR spectra of isolated malvidin-3-*p*-coumaroyl-glucoside from grape pomace. The chemical shifts obtained from ^{13}C NMR from coumaroyl group δ 168.83 ($R1CO_2R2$), δ 114.17 ($CH{=}CH_2CO_2R$), aglycon moiety δ 159.84 (C9), δ 158.73 (C2), δ 158.18 (C5), δ 149.77 (C5'), δ 145.20 (C3), δ 119.78 (C1'), δ 113.54 (C10), δ 110.72 (C2') δ 57.28 (OCH_3) and glucopyranoside moiety δ 131.15 (G1, G6), δ 126.98 (G1), δ 116.86 (G3, G5), δ 76.16 (G5), δ 75.01 (G2), δ 71.79 (G4) were in good accordance with the ^{13}C NMR spectra of malvidin-3-glucoside published by Andersen *et al*. (1995), Atanasova *et al*. (2002) and Aguirre *et al*. (2010). The most significant shift of signal from 62 ppm for G6 to 64.34 ppm was the proof of the attached *p*-coumaroyl group on position 6 of the glucose.

Also, the coil fraction coil of "Vranec" grape pomace was enriched by a minor quantity of malvidin-3-*O*-(6"-acetyl)-glucoside. This pigment was tentatively identified by its MS fragmentation (Fig. 68).

The presence of acetylvitisin B in traces at 39.3 min was tentatively identified in the "Merlot" coil fraction (Fig. 69).

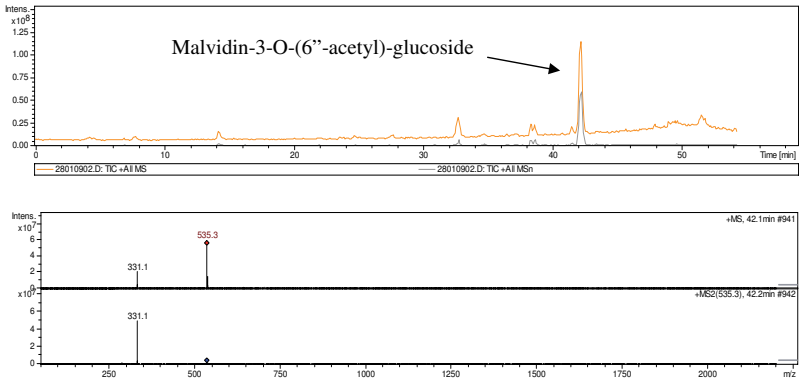


Fig. 68. Total ion chromatogram (TIC) and mass spectrometric data of malvidin-*p*-acetyl-glucoside in fraction 4 of "Vranec" grape pomace

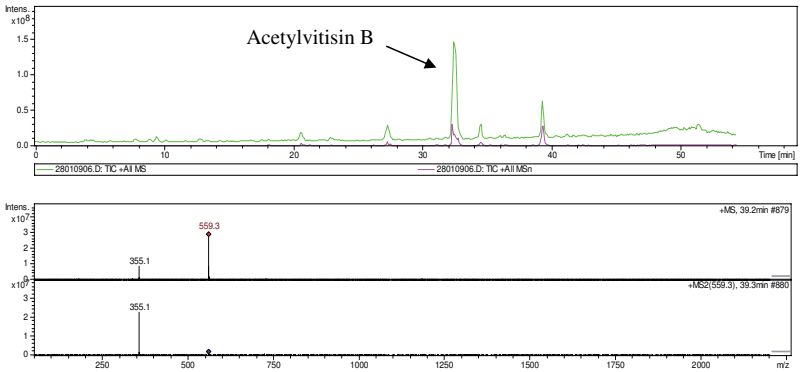


Fig. 69. Total ion chromatogram (TIC) and mass spectrometric data of acetylvitisin B in fraction 2 from "Merlot" before purification

The order of elution of anthocyanins-3-*O*-glycosides were the same in the three varieties of grape pomace. Delphinidin-3-*O*-glucoside is eluted first in the "CCC" chromatogram. The substitution of three hydroxyl groups (-OH) in B ring (Fig. 49) enables the highest polarity of this pigment in comparison with the other isolated pigments. During the "CCC" elution, the lower (more polar) phase was used as the mobile phase. In the "head-to-tail" mode the most polar anthocyanins will elute first. Petundin-3-*O*-glucoside was the second isolated pigment in the first "CCC" fraction F1. This anthocyanin-3-*O*-glucoside is less polar compared with delphinidin-3-*O*-glucoside since in its B ring at position R_1 (Fig. 49) is substituted by a methoxy group which is less polar than the hydroxyl group.

The most abundant pigment malvidin-3-*O*-glucoside was isolated from fraction F2.

Fraction F3 was a mixture of two substituted anthocyanins cyandin-3-*O*-glucoside and peonidin-3-*O*-glucoside. The first one was cyanidin-3-*O*-glucoside being more polar with two hydroxyl groups in B ring in comparison with peonidin-3-*O*-glucoside with a hydroxyl and a methoxy group (Fig. 49).

The order of elution of anthocyanins depends on many factors such as polarity of substituents in B-rings and solvent systems. The experiments of separation of anthocyanins-3-*O*-glucoside from grape pomaces by using three coil high speed CCC system confirmed that the order of separation mainly depends on the degree of hydroxyl substitution in the B ring. Furthermore, the order of the elution of the pigments with the same number of substituted groups depends on their polarity. The findings in this dissertation are in accordance with the findings of Salas *et al*. (2005) who

confirmed the same order of separation in the "head-to-tail" mode of CCC separation.

Separation of anthocyanins from "Merlot" grape pomace was enriched with other two pigments in comparison with "Vranec" and "Merlot" grape pomace. In the coil fraction of "Merlot" grape pomace a small quantity of malvidin-3-*p*-acetyl-glucoside and acetylvitisin B was detected. These results are in good agreement with the findings of Renault *et al*. (1997) who explained that acylated anthocyanins are eluting before the coumaroyl anthocyanins.

Table 10 shows the amount of pure pigments (in mg) isolated and purified from 1.0 g of crude antocyanin extract from "Pinot noir", "Merlot" and "Vranec" variety of grape pomace. According to the presented results, it is obvious that the skin and seeds of grapes after 20 days maceration time still contained significant amount of malvidin-3-gucoside and malvidin-3-*p*-coumaroyl glucoside. The other isolated anthocyanins were present in very small quantities.

Comparison of the results reveals that the highest amount of anthocyanins is obtained from "Vranec" grape pomace. Furthermore, this grape pomace contained the highest quantities of malvidin-3-glucoside and malvidin-3-p-coumaroyl-glucoside which explain the violet-blue color of this variety.

Table 10. Isolated anthocyanins from "Pinot Noir", "Merlot" and "Vranec" grape pomace

Variety	*Fraction*	*Rt (min.)*	*Molecular ion M^+(m/z)*	*Fragment ion (MS^2)*	*Structure*	*Amount of pure compound (mg)*
Pinot noir	1	15.0	465.3	303.0	Delphinidin-3-glucoside	0.2
	1	19.5	449.4	287.0	Cyanidin-3-glucoside	0.7
	2	28.0	493.4	331.0	Malvidin-3-glucoside	10.0
	3	22.0	479.2	317.0	Petunidin-3-glucoside	0.8
	3	25.9	463.3	301.0	Peonidin-3-glucoside	0.6
	Coil fraction	44.7	639.5	331.0	Malvidin-3-p-coumaroyl-glucoside	0.9
Merlot	1	15.0	465.3	303.0	Delphinidin-3-glucoside	0.3
	1	19.5	449.4	287.0	Cyanidin-3-glucoside	2.1
	2	28.0	493.4	331.0	Malvidin-3-glucoside	14.2
	3	22.0	479.2	317.0	Petunidin-3-glucoside	0.9
	3	25.9	463.3	301.0	Peonidin-3-glucoside	2.7
	Coil fraction	44.7	639.5	331.0	Malvidin-3-p-coumaroyl-glucoside	4.2

Table 10. Isolated anthocyanins from "Pinot Noir", "Merlot" and "Vranec" grape pomace (Cont'd)

Variety	*Fraction*	*Rt (min.)*	*Molecular ion M^+(m/z)*	*Fragment ion (MS^2)*	*Structure*	*Amount of pure compound (mg)*
Vranec	1	15.0	465.3	303.0	Delphinidin-3-glucoside	0.4
	1	19.5	449.4	287.0	Cyanidin-3-glucoside	3.3
	2	28.0	493.4	331.0	Malvidin-3-glucoside	19.0
	3	22.0	479.2	317.0	Petunidin-3-glucoside	0.8
	3	25.9	463.3	301.0	Peonidin-3-glucoside	1.4
	Coil fraction	44.7	639.5	331.0	Malvidin-3-p-coumaroyl-glucoside	5.5

3.3.1. Visual detection thresholds and "color activity concept" of isolated anthocyanins

The color contribution of the isolated anthocyanins to the overall color of red wines was subject of studies of Degenhardt *et al*. (2000a). According to the results, the most abundant anthocyanin was malvidin-3-glucoside (500 mg/L of pure malvidin-3-glucoside per liter of red wine) which contributes predominantly to the color of red wines.

On the other hand, the contribution of other pigments to the overall color of pomace was different for each grape variety. Table 11 presents the visual detection thresholds of isolated anthocyanin-3-glucosides and the

corresponding color activity values (CAVs). It is notable that delphindin-3-glucoside did not contribute to the overall color of grape pomace.

Table 11. Visual detection thresholds and Color activity values of isolated anthocyanins

Isolated anthocyanins	*Visual detection thresholds (mg/L)*	*Color activity value (CAV) for Pinot Noir grape pomace*	*Color activity value (CAV) for Merlot grape pomace*	*Color activity value (CAV) for Vranec grape pomace*
Dephinidin-3-glucoside	2	<1	<1	<1
Cyanidin-3-glucoside	1	<1	2.1	3.3
Petunidin-3-glucoside	0.2	4.0	4.5	4.0
Peonidin-3-glucoside	0.25	2.4	10.8	5.6
Malvidin-3-glucoside	1.25	8.0	11.3	15.2
Malvidin-3-p-coumaroyl-glucoside	0.75	1.2	5.6	7.3

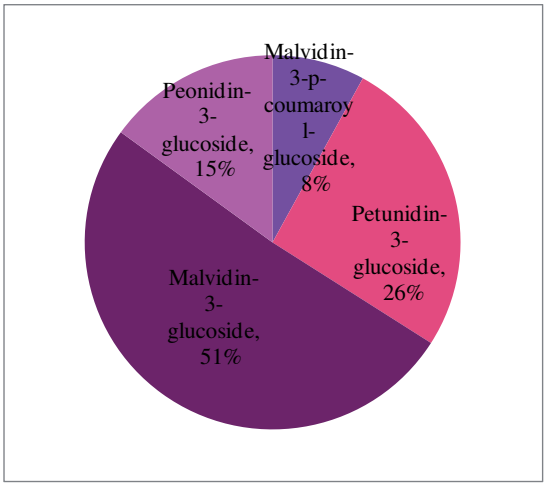


Fig. 70. Particular contribution of the monomeric anthocyanins-3-glucoside to the overall color of "Pinot Noir" grape pomace

However, petunidin-3-glucoside contributed more significant to the color of "Pinot Noir" grape variety in comparison with its contribution to the other two varieties. As it is presented in Fig. 70 petunidin-3-glucoside participated with 26 % of the total monomeric anthocyanins-3-glucoside. On the other hand, the color activity value of malvidin-3-*p*-coumaroyl-glucoside, responsible for blue color, contributes only with 8 % in "Pinot Noir" grape pomace in comparison with "Merlot" and "Vranec" grape pomace where the contribution was higher. This might be the reason for the deeply red color of "Pinot Noir" grape variety in comparison with the red violet and violet-blue color of "Merlot" and "Vranec" grape pomace, respectively.

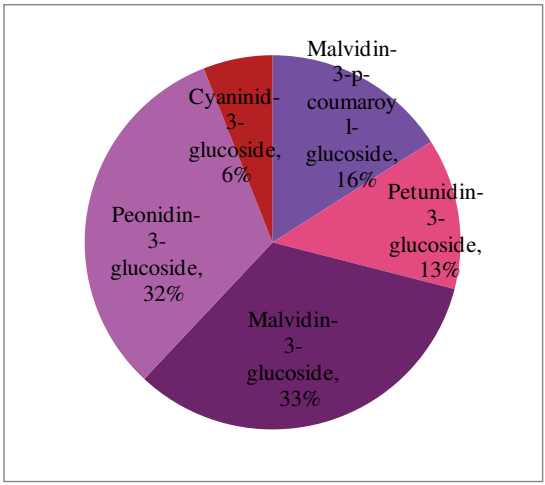


Fig. 71. Particular contribution of the monomeric anthocyanins-3-glucoside to the overall color of "Merlot" grape pomace

Contribution of cyanidin-3-glucoside and petunidin-3-glucoside in "Merlot" grape pomace is significant for the red shade of this grape variety. However, the higher particular contribution of peonidin-3-glucoside and malvidin-3-glucoside of above 30 % might be the reason for the deeply violet color of this grape variety.

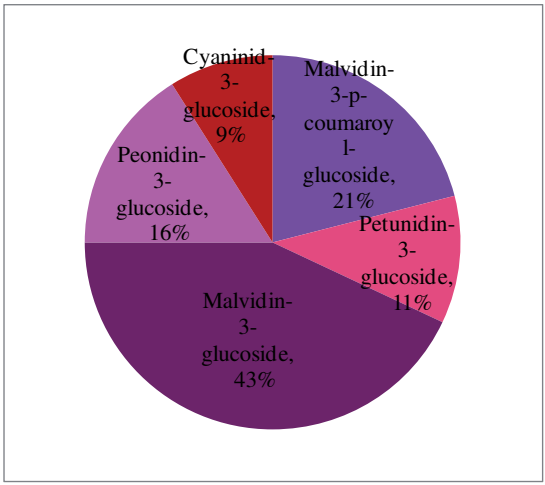


Fig. 72. Particular contribution of the monomeric anthocyanins-3-glucoside to the overall color of "Vranec" grape pomace

The highest particular contribution of malvidin-3-*p*-coumaroyl-glucoside with percentage of 21 % was responsible for violet-blue color of "Vranec" grape pomace in comparison with red-violet color of "Merlot" grape pomace. This finding can be explained by the intramolecular interactions of anthocyanins and other polyphenolics such as *p*-coumaric acid.

3.4. Effect of enzyme treatment on volatile profile of different varieties of white and red wines from Macedonia by using HS-SPME-GC/MS

The last chapter of this dissertation describes the volatile profile of commercial white and red wines from Macedonia. The effect of enzyme treatment with "Endozym Aromatic" and "AR 2000" on the volatile fraction of wines from different varieties will also be discussed in this chapter.

The most cultivated variety of white grapes in the territory of Macedonia is "Muscat de Frontignan". Therefore, nine samples of wines known as "Temjanika or Muscat" produced from "Muscat de Frontignan" grape variety were object of this study. These commercial wines selected from different wineries and different years of production were produced by the standard wine-making procedure including the application of the enzyme "Endozym Aromatic" for liberation of glucosidically bound compounds in order to enrich the smell and the taste of the produced wines. The vinification procedure described in the Materials and Methods part includes cold macerations at 12-14°C during alcoholic fermentation. Application of enzyme during the vinification procedure is necessary because its two functions by its pectolytic activity it allows a better extraction of free aromatic compounds and by its β-glycosidasic activity it is acting on aroma precursors.

The extraction of free volatiles was performed by application of solid phase microextraction in head-space mode (HS-SPME). Regarding selection of the fibers, the 50/30 μm Carbowax-Divinylbenzene-Polydimethylsiloxan (CRB-DVB-PDMS) fiber was more sensitive than the 85 μm polyacrylate fiber. This is depicted in the chromatograms in Fig. 73 and 74. The area of peaks of the total ion chromatogram presented in Fig. 73 is few times

higher than area of peaks from extracted volatiles on the same "Temjanika" wine (V8) by using 85 μm polyacrylate fiber (Fig. 74). In head-space mode, CRB-DVB-PDMS had better response for extraction of volatiles such as terpenes, alcohols, esters and acids in comparison with polyacrylate fiber.

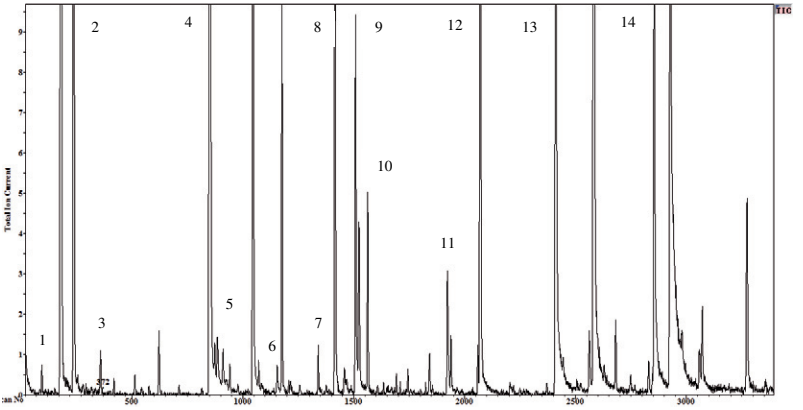


Fig. 73. GC-MS total ion chromatogram of "Temjanika" (V8) white wine extracted by HS-SPME using 50/30 μm CRB-DVB-PDMS (Carbowax-Divinylbenzene-Polydimethylsiloxane) fiber
Peak identification: 1 limonene, 2 ethyl hexanoate, 3 hexylacetate, 4 ethyl octanoate, 5 furfural, 6 linalool, 7 hotrienol, 8 ethyl decanoate, 9 diethyl succinate, 10 α-terpineol, 11 hexanoic acid, 12 phenylethyl alcohol, 13 octanoic acid, 14 decanoic acid

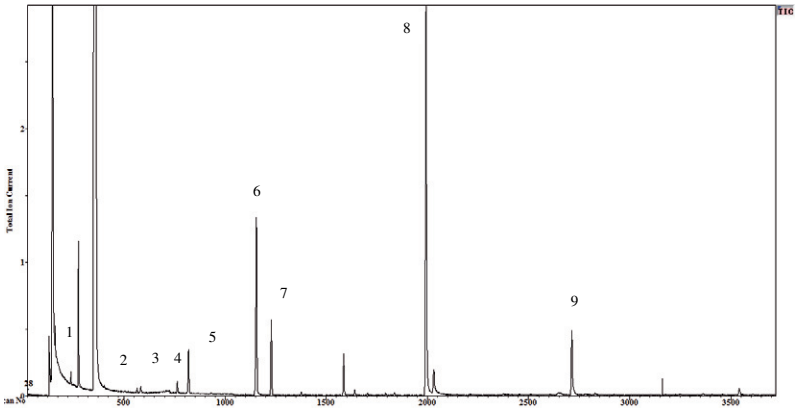


Fig. 74. GC-MS total ion chromatogram of "Temjanika" (V8) white wine extracted by HS-SPME using 85 μm polyacrylate fiber. Peak identification: 1 ethyl acetate, 2 ethyl butyrate, 3 propanol, 4 isobutyl alcohol, 5 isoamyl acetate, 6 3-methyl-1-butanol, 7 ethyl hexanoate, 8 ethyl octanoate, 9 ethyl decanoate

The volatile profile of Temjanika and Muscat wines (V1-V9) produced from "Muscat de Frontignan" grape variety with application of enzyme "Endozym Aromatic" is presented in Table 12.

For quantification of volatile compounds 2-heptanol was used as internal standard in concentration of 4 μg/L. The semiquantitative analyses were carried out assuming an equal response factor. For this purpose 50μL of 2-heptanol was diluted in 100 mL of diethyl ether. 50 μL of internal standard solution was added into SPME vials containing 2 mL of wine. All analysis was performed in duplicate and the results were expressed as mg and μg of compound per litre of wine. The same temperature gradient used for MS identification was applied for quantification of volatile components in the wines.

Table 12. Mean concentrations of free varietal compounds (mg/L) and relative standard deviations (n = 2) of Temjanika and Muscat wines (V1-V9) from Macedonia

Components	***V1***	***V2***	***V3***	***V4***	***V5***
Terpenes					
p-Limonene	0.21±0.05	1.35±0.33	1.12±0.16	1.38±0.42	0.22±0.03
γ-Terpinene	0.00	0.35±0.02	0.00	0.00	0.00
p-Cymene	0.00	0.10±0.02	0.00	0.00	0.00
α-Terpineol	0.00	0.11±0.03	0.62±0.04	0.00	0.00
α-Terpinolene	0.71±0.02	0.72±0.23	0.00	0.00	0.00
Linalool	0.00	0.00	1.50±0.16	0.57±0.02	3.56±0.29
Geraniol	0.00	0.00	0.16±0.02	0.00	0.37±0.01
Hotrienol	0.00	0.00	0.00	0.00	0.35±0.01
Total terpenes	**0.92**	**2.63**	**3.40**	**1.95**	**4.50**
Alcohols					
Phenylethyl alcohol	7.52±0.24	2.75±0.33	2.78±0.04	2.65±0.79	11.22±0.69
Aldehydes					
Furfural	0.00	0.00	0.00	0.00	0.43±0.01
Esters					
Ethyl hexanoate	0.98±0.63	0.96±0.09	0.78±0.31	3.90±0.10	1.83±0.96
Hexyl acetate	1.46±0.08	0.68±0.09	0.29±0.07	0.00	0.34±0.01
Ethyl octanoate	2.49±0.66	2.65±1.81	8.57±0.51	1.65±0.27	3.73±1.18
Ethyl decanoate	6.39±0.45	0.00	1.62±0.24	2.49±0.06	7.46±0.45
Diethyl succinat	0.00	0.00	0.70±0.29	0.00	3.67±0.28
Phenylethyl acetate	0.00	0.00	0.00	0.00	0.43±0.05

Table 12. Mean concentrations of free varietal compounds (mg/L) and relative standard deviations (n = 2) of Temjanika and Muscat wines (V1-V9) from Macedonia (Cont'd)

Components	***V1***	***V2***	***V3***	***V4***	***V5***
Total esters	**11.32**	**4.29**	**11.96**	**8.04**	**17.46**
Ketones					
2,3 Butadienone	0.53±0.02	0.00	0.59±0.03	0.57±0.00	0.00
Acids					
Hexanoic acid	0.00	0.73±0.02	0.61±0.00	0.53±0.09	1.50±0.07
Octanoic acid	2.21±0.24	1.58±0.24	1.55±0.69	0.47±0.12	2.35±1.45
Decanoic acid	2.47±0.36	0.75±0.09	0.63±0.05	2.97±0.68	0.81±0.09
Total acids	**4.68**	**3.06**	**2.79**	**3.97**	**4.66**
Components	***V6***	***V7***	***V8***	***V9***	
Terpenes					
p-Limonene	2.17±0.41	0.79±0.63	1.28±0.36	2.16±0.45	
γ-Terpinene	1.25±0.03	0.22±0.01	0.00	0.00	
p-Cymene	0.30±0.06	0.00	0.00	0.00	
α-Terpineol	0.54±0.00	0.45±0.03	1.79±0.13	0.16±0.02	
β-Citronellol	0.00	0.00	0.39±0.03	0.00	
α-Terpinolene	0.43±0.02	0.00	0.00	0.58±0.23	
Linalool	0.00	0.42±0.03	6.60±1.24	0.00	
Geraniol	0.00	0.00	0.98±0.05	0.00	
Hotrienol	0.00	0.00	0.51±0.05	0.00	
Total terpenes	**4.69**	**1.88**	**11.55**	**2.90**	

Table 12. Mean concentrations of free varietal compounds (mg/L) and relative standard deviations (n = 2) of Temjanika and Muscat wines (V1-V9) from Macedonia (Cont'd)

Components	***V6***	***V7***	***V8***	***V9***
Alcohols				
Phenylethyl alcohol	2.36±0.62	3.03±0.34	13.60±1.34	1.19±0.24
Aldehydes				
Furfural	0.00	0.00	0.23±0.03	0.53±0.29
Esters				
Ethyl hexanoate	0.76±0.25	0.63±0.05	1.86±0.21	0.92±0.56
Hexyl acetate	0.00	0.00	1.41±0.05	2.46±0.09
Ethyl octanoate	2.49±0.60	1.03±0.07	4.08±1.87	5.71±1.02
Ethyl decanoate	0.00	1.17±0.02	7.31±1.02	1.05±0.45
Diethyl succinat	1.22±0.02	1.56±0.11	1.15±0.23	0.00
Phenylethyl acetate	0.00	0.00	1.48±0.04	0.00
Total esters	**4.47**	**4.39**	**17.29**	**10.14**
Ketones				
2,3 Butadienone	0.49±0.04	0.42±0.08	0.00	0.00

Table 12. Mean concentrations of free varietal compounds (mg/L) and relative standard deviations (n = 2) of Temjanika and Muscat wines (V1-V9) from Macedonia (Cont'd)

Components	***V6***	***V7***	***V8***	***V9***
Acids				
Hexanoic acid	0.51±0.03	0.54±0.01	1.50±0.16	0.77±0.05
Octanoic acid	2.26±1.07	1.07±0.23	3.15±0.93	1.06±0.69
Decanoic acid	0.58±0.22	2.58±0.24	2.35±0.67	5.58±0.39
Total acids	**3.35**	**4.19**	**7.00**	**7.41**

Fig. 75 shows the histogram of the total terpenes, alcohol, esters and acids in wines produced from "Muscat de Frontignan" grape variety. Esters were the most predominant volatile compounds in most of the analyzed wines. The highest concentrations of esters were observed for wine V5 from the 2008 vintage and the highest concentration of terpenes and alcohols were quantified in wine V8 from the 2007 vintage. The largest abundance of acids was observed in wine V9 from the 2006 vintage. The lowest concentrations of total volatiles were extracted from wines V6 and V7 from 2007 and 2006 vintage.

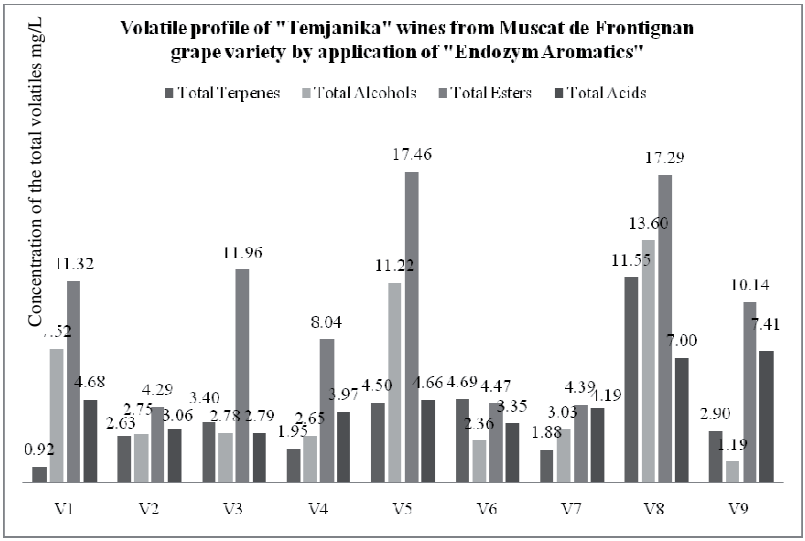


Fig. 75. Histogram of the free volatiles in wines from "Muscat de Frontignan" grape variety

One sample of white wine from the other six selected commercial wines was produced from "Muscat Ottonel"- the least abundant grape variety on the territory of Macedonia. Its wine-making procedure is explained in the "Material and Methods" part. The vinification procedure of this wine included cold maceration at 10 °C for 9 h. However, this wine is not enzymatically treated. This wine sample together with the other selected wines from Riesling, Chardonnay, Vranec, Merlot and Cabernet Sauvignon grape varieties were enzymatically treated with "AR 2000" in order to liberate the glycosidically bound volatile compounds responsible for the aromatic profile of the wines.

The volatile compounds of control and treated wines (V10-V15) produced from white and red grape varieties with enzyme "AR 2000" are depicted in Table 13.

Table 13. Mean concentrations of bound varietal compounds and relative standard deviations (n = 2) of white and red wines (V10-V15) from Macedonia

Components	*V10 control wine (mg/L)*	*V10 treated wine (μg/L)*	*V11 control wine (mg/L)*
Terpenes			
α-Terpineol	0.00	0.00	0.00
β-Citronellol	0.00	0.00	0.00
Nerol	0.00	0.00	0.00
Geraniol	0.00	0.00	0.00
Linalool	0.00	0.00	0.00
Hotrienol	3.25±1.43	312.23±1.54	0.00
Total terpenes	**3.25**	**312.23**	**0.00**
Alcohols			
2-Methyl propanol	1.28±0.04	0.00	1.85±0.63
3-Methylbutanol	23.46±2.05	25740.2±70.2	48.23±9.11
Hexanol	1.45±0.09	3512.8±23.72	54.23±0.027
Benzyl alcohol	0.00	691.3±43.78	0.00
Phenylethyl alcohol	4.68±1.00	918.9±12.58	1.89±0.22
Total alcohols	**30.87**	**30863.2**	**106.20**
Esters			
Ethyl butyrate	0.70±0.02	0.00	0.70±0.36
Isoamyl acetoacetate	1.14±0.04	0.00	1.21±0.31
Ethyl hexanoate	1.98±0.12	0.00	1.12±0.57
Hexyl acetate	2.21±0.87	0.00	0.32±0.09
Ethyl octanoate	8.47±0.91	0.00	1.37±0.27
Ethyl decanoate	3.52±0.80	0.00	1.51±0.51
Diethyl succinate	0.54±0.07	0.00	0.47±0.25
Total esters	**18.56**	**0.00**	**6.70**
Aldehydes			
Furfural	0.98±0.02	0.00	1.24±0.17
Benzaldehyde	0.00	21.89±0.47	0.00
	0.98	**21.89**	**1.24**
Acids			
Acetic acid	0.57±0.02	43.32±17.23	0.69±0.19
Lactic acid	0.59±0.17	0.00	1.08±0.09
Hexanoic acid	0.56±0.17	0.00	0.00
Octanoic acid	4.61±0.66	1216.1±24.74	2.11±0.09
Decanoic acid	0.00	207.81±14.71	0.00

Table 13. Mean concentrations of bound varietal compounds and relative standard deviations (n = 2) of white and red wines (V10-V15) from Macedonia (Cont'd)

Components	***V10 control wine (mg/L)***	***V10 treated wine (μg/L)***	***V11 control wine (mg/L)***
Geranic acid	0.00	374.32±13.01	0.00
Total acids	**6.33**	**1841.62**	**3.88**
Phenols			
4-Vinyl guaiacol	0.00	0.00	0.00
Components	***V13 control wine (mg/L)***	***V13 treated wine(μg/L)***	***V14 control wine (mg/L)***
Alcohols			
2-Methyl propanol	4.32±1.86	0.00	4.61±0.43
3-Methylbutanol	43.42±2.92	0.00	68.27±1.79
2-Propanol	0.31±0.02	0.00	0.55±0.35
Hexanol	1.09±0.10	53.57±0.31	1.47±0.72
Benzyl alcohol	0.00	143.23±13.98	0.00
Phenylethyl alcohol	6.93±0.23	1113.2±71.61	11.92±1.43
Total alcohols	**56.07**	**1310.02**	**86.82**
Esters			
Ethyl acetate	3.81±0.02	0.00	0.00
Isoamyl acetoacetate	0.00	61.27±0.42	0.00
Ethyl hexanoate	1.07±0.08	0.00	0.15±0.07
Ethyl octanoate	1.83±0.31	0.00	4.97±0.21
Diethyl succinate	3.26±0.27	55.13±0.07	2.78±0.97
Total esters	**9.97**	**116.40**	**7.90**
Acids			
Lactic acid	4.30±0.23	0.00	3.14±0.57
Acetic acid	1.78±0.29	0.00	7.25±1.29
Octanoic acid	0.20±0.12	213.64±19.27	0.10±0.07
Decanoic acid	0.00	50.34±7.59	0.00
Total acids	**6.28**	**263.98**	**10.49**
Phenols			
4-Vinyl phenol	0.00	0.00	0.00

Table 13. Mean concentrations of bound varietal compounds and relative standard deviations (n = 2) of white and red wines (V10-V15) from Macedonia (Cont'd)

Components	*V13 control wine (mg/L)*	*V13 treated wine(μg/L)*	*V14 control wine (mg/L)*
Others			
Dihydro-3-methylen-2,5 -furandione	3.94±0.10	59.37±10.75	0.00
Vinylfuran	0.00	5.98±0.09	0.00
Components	***V11 treated wine (μg/L)***	***V15 control wine (mg/L)***	***V15 treated wine (μg/L)***
Terpenes			
α-Terpineol	0.00	1.27±0.13	230.91±23.62
β-Citronellol	0.00	0.00	56.48±15.43
Nerol	0.00	0.00	1688.2±46.49
Geraniol	19.21±0.02	0.00	1526.4±65.23
Linalool	0.00	1.80±0.04	90.23±17.84
Hotrienol	0.00	1.63±0.72	350.12±29.3
Total terpenes	**19.21**	**4.70**	**3942.34**
Alcohols			
2-Methyl propanol	0.00	0.83±0.27	0.00
3-Methylbutanol	126.32±24.71	12.89±1.94	145.37±29.54
Hexanol	647.41±13.23	12.11±0.52	52.87±13.07
Benzyl alcohol	160.75±30.02	0.00	244.23±91.43
Phenylethyl alcohol	518.21±16.61	6.35±0.13	384.91±11.64
Total alcohols	**1452.69**	**32.18**	**827.38**
Esters			
Ethyl butyrate	0.00	0.54±0.07	0.00
Isoamyl acetoacetate	0.00	0.00	0.00
Ethyl hexanoate	0.00	3.94±0.52	0.00
Hexyl acetate	0.00	0.48±0.03	0.00
Ethyl octanoate	0.00	3.62±1.09	0.00
Ethyl decanoate	0.00	3.02±0.65	0.00
Diethyl succinate	0.00	2.82±0.31	0.00
Total esters	**0.00**	**14.42**	**0.00**

Table 13. Mean concentrations of bound varietal compounds and relative standard deviations (n = 2) of white and red wines (V10-V15) from Macedonia (Cont'd)

Components	***V11 treated wine (μg/L)***	***V15 control wine (mg/L)***	***V15 treated wine (μg/L)***
Aldehydes			
Furfural	0.00	1.33±0.06	0.00
Benzaldehyde	37.23±0.02	0.00	47.24±13.92
	37.23	**1.33**	**47.24**
Acids			
Acetic acid	46.94±14.78	0.96±0.06	26.39±11.95
Lactic acid	0.00	0.38±0.01	0.00
Hexanoic acid	45.12±0.03	0.00	30.93±3.85
Octanoic acid	589.34±78.91	2.13±0.12	635.71±24.97
Decanoic acid	588.24±0.04	0.00	423.93±51.98
Geranic acid	0.00	0.00	651.95±20.49
Total acids	**1269.64**	**3.47**	**1768.91**
Phenols			
4-Vinyl guaiacol	0.00	4.14±0.08	0.00
Components	***V14 treated wine (μg/L)***	***V12 control wine (mg/L)***	***V12 treated wine (μg/L)***
Alcohols			
2-Methyl propanol	0.00	12.71±5.06	0.00
3-Methylbutanol	0.00	10.17±1.29	0.00
2-Propanol	0.00	0.80±0.26	0.00
Hexanol	91.23±21.02	2.03±0.26	64.53±5.39
Benzyl alcohol	79.29±27.35	0.00	0.00
Phenylethyl alcohol	1521.2±15.3	2.96±6.46	2622.1±34.92
Total alcohols	**1691.74**	**28.67**	**2686.92**
Esters			
Ethyl acetate	0.00	28.45±3.23	277.12±26.43
Isoamyl acetoacetate	0.00	2.53±0.87	1202.2±20.49
Ethyl hexanoate	0.00	4.93±1.57	0.00
Ethyl octanoate	0.00	1.45±0.43	0.00
Diethyl succinate	62.98±27.24	5.80±0.87	43.28±27.99
Total esters	**62.98**	**43.16**	**1522.62**
Acids			
Lactic acid	0.00	7.09±1.13	171.09±0.02
Acetic acid	0.00	2.75±0.45	635.67±0.18

Table 13. Mean concentrations of bound varietal compounds and relative standard deviations (n = 2) of white and red wines (V10-V15) from Macedonia (Cont'd)

Components	***V14 treated wine (μg/L)***	***V12 control wine (mg/L)***	***V12 treated wine (μg/L)***
Octanoic acid	1040.2±65.23	0.92±0.08	217.77±23.44
Decanoic acid	83.29±16.97	0.00	423.93±51.98
Total acids	**1123.51**	**10.76**	**1448.46**
Phenols			
4-Vinyl phenol	347.99±31.82	0.00	24.79±7.28
Others			
Dihydro-3-methylen-2,5 -furandione	276.17±31.98	0.00	7971.1±2.40
Vinylfuran	0.00	0.00	0.00

Results in Fig. 76 present the total abundance of the most dominant free volatiles in control wines V10-V15. From the histogram depicted in Fig. 76 the most abundant volatiles were alcohols with exception of wine V12 from the "Vranec" variety were the most abundant group of volatile compounds were esters. The largest concentration of alcohols (106.20 mg/L) was extracted from wine produced from Chardonnay grape variety. However, this level did not exceed the critical level of 400 mg/L when hexanol can produce unpleasant flavour (Gómez García-Carpintero *et al*., 2011).

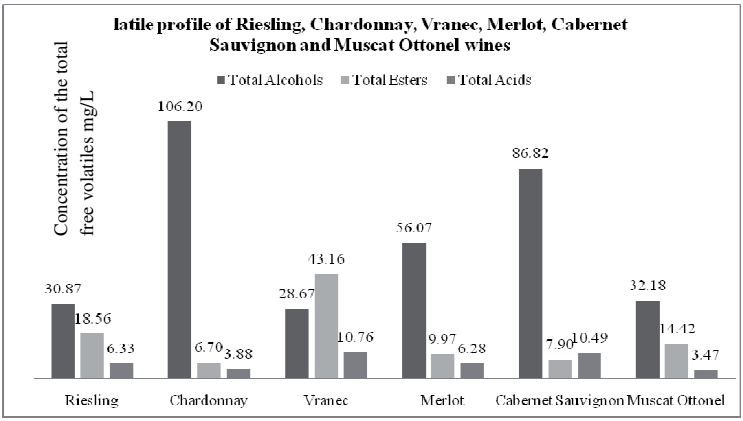


Fig. 76. Histogram of the concentration of free volatiles in Riesling, Chardonnay, Vranec, Merlot, Cabernet Sauvignon and Muscat Ottonel wines

The histogram presented in Fig. 77 shows the concentrations of liberated bound volatiles after application of enzyme "AR 2000". It is notable that the effect of enzyme is the highest with regard to the release of alcohols in all samples except for "Muscat Ottonel" wine were the enzymes had the highest impact on the release of acids.

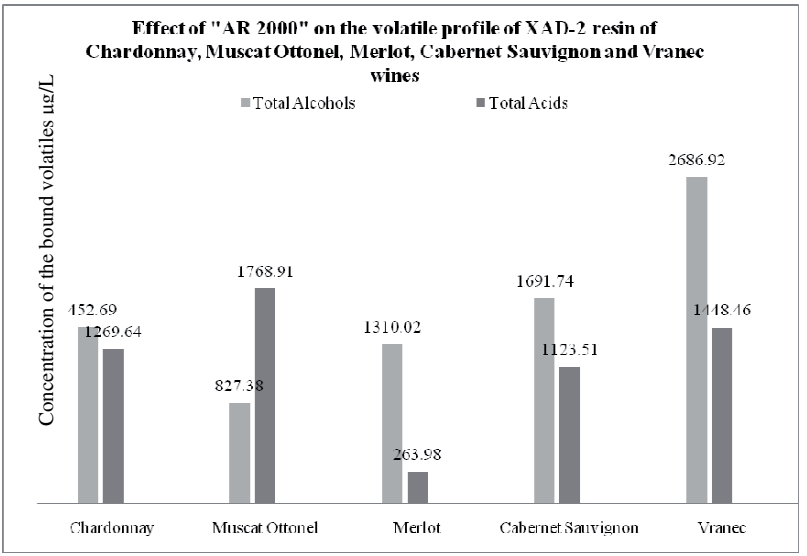


Fig. 77. Histogram of the concentration of bound volatiles of Chardonnay, Muscat Ottonel, Merlot, Cabernet Sauvignon and Vranec wines

Results depicted in Fig. 76 and 77 indicated strong dependence of the volatile compounds to the enzymatic treatment and variety of grapes. From the results in the text below it can be concluded that enzymatic treatment by application of "Endozyme Aromatic" affects most of the group of terpenes in Muscat variety. On the other hand, application of "AR 2000" affects most of the alcohols and acids in the other treated wines.

3.4.1. Terpenes

Terpenes are one of the most important group of compounds which contribute to the overall aroma of Muscat wines (Kang *et al*., 2010). Limonene is one of the most important monoterpene responsible for the characteristic "citrus-like" taste of "Temjanika" wines formed by

cyclisation of geranyl pyrophosphate. Limonene was the most abundant monoterpene in wine samples produced from "Muscat de Frontignan" grapes with concentrations from 0.22 to 2.17 mg/L (Table 12).

Apart from the high abundance of limonene, significant concentrations of other terpenes such as γ-terpinene, p-cymene, α-terpinolene, α-phellandrene and α-terpineol in wines V6 and V9 were also detected. Their concentrations estimated by head space solid phase microextraction and gas chromatography mass spectrometry (HS-SPME/GC-MS) were in good agreement with the published results from for Muscat grapes by Kang *et al*. (2010).

Furthermore, the concentration of *α*-terpineol in Muscat wines from Macedonia was in good agreement with its abundance in white wines from Poland extracted in similar procedure by application of SPE-SPME-GC/MS (Dziadas & Jelén, 2010). Cyclisation reaction of other terpenes such as nerol, geraniol and linalool in acid conditions can produce α-terpineol. The relatively high concentration in Temjanika wines (0.11-0.62 mg/L) can be the result of overripe grapes. A similar concentration of α-terpineol (196 μg/L) in sweet Fiano wines was observed as result of overripe grapes by Genovese *et al*. (2007).

Citronellal detected in some wines can either be a product of metabolite activity of yeast or it can be synthesized from nerol and geraniol (Sánchez Palomo *et al*., 2007).

Hotrienol is the most abundant terpene in "Muscat" grapes and wines. This terpene alcohol is formed by reaction of elimination of water from 2,6-dimethyl-3,7-octadiene-2,6-diol (Sánchez Palomo *et al*., 2006). Significant effect of "AR 2000" on the concentration of hotrienol is notable in wines produced from "Riesling" and "Muscat Ottonel" (Fig. 78).

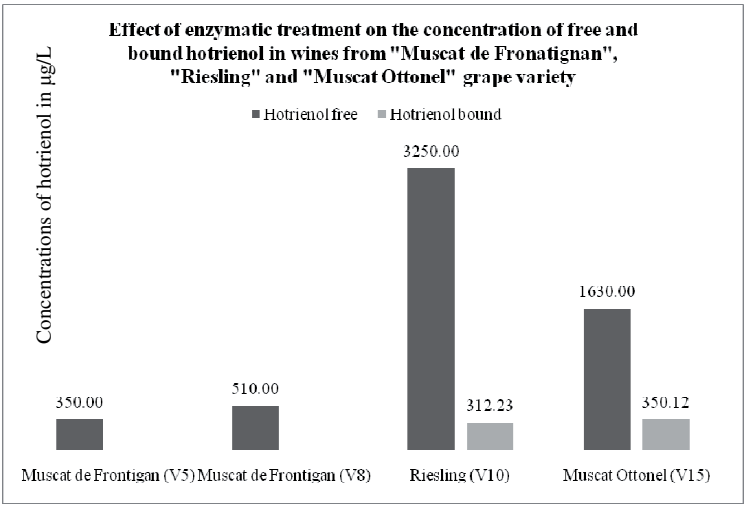


Fig. 78. Histogram of the concentration of free and bound hotrienol in wines from "Muscat de Frontignan", "Riesling" and "Muscat Ottonel" grape variety

The histogram depicted in Fig. 78 shows the concentrations of free and bound hotrienol in wines produced from "Muscat de Frontignan", "Riesling" and "Muscat Ottonel" grape variety. More precisely, wines V5 and V8 produced from "Muscat de Fronatignan" grape variety and treated with "Endozyme Aromatic" during winemaking contained 350 µg/L and 510 µg/L of total hotrienol (Table 12). On the other hand, enzymatically treated wines V10 and V15 produced from "Riesling" and "Muscat Ottonel" grape varieties released 312.23 µg/L and 350.12 µg/L only from bound forms (Table 13). This difference can be the result of different varieties of grapes as well as different effects of the applied enzymes.

Geraniol and nerol are the most important terpenes responsible for the fruity flavor of white wines. Geraniol was quantified in bound form in wine V11 produced from Chardonnay grape variety in the lowest concentration of 19.21 µg/L. The concentrations of the same monoterpene in some wines

produced from "Muscat de Frontignan" grapes were in the range of 160 to 980 μg/L. However, the highest concentrations of the monoterpenes geraniol and nerol were detected in bound form in "Muscat Ottonel" wine V15. The concentrations of geraniol and nerol in treated wine V15 were 1526 μg/L and 1688 μg/L, respectively. From those results we can conclude that high levels of geraniol and nerol are present as glycosides which were cleaved after treatment with "AR 2000" since not detectable quantities of the two monoterpenes were observed in control V15 wine. The result for the liberated level of geraniol was in the same range (1622 μg/L) in Muscat grapes released after application of the same enzyme "AR 2000" as reported by Kang *et al*. (2010). Furthermore, β-citronellol was quantified only in the sample of "Muscat Ottonel" in bound form in concentration of 56.48 μg/L. Results for linalool in Table 12 and 13 confirmed the presence of this monoterpene only in wines produced from "Muscat" varieties of grapes.

From the results shown in Table 12 and 13 it is obvious that the relatively high abundance of terpenes in wines V1-V9 (produced from "Muscat de Frontignan") and wine V15 (from "Muscat Ottonel") in comparison with the other analyzed wines is characteristic for the aroma of "Muscat" cultivars.

3.4.2. Alcohols

Alcohols are the largest group of compounds which are formed by the metabolitic activity of yeasts during alcoholic fermentation (Gómez García-Carpintero *et al*., 2011). If we compare the quantities of total alcohols in wines produced from "Muscat de Frontignan" by "Endozyme Aromatic" and wines from Riesling, Muscat Ottonel, Merlot, Vranec and

Cabernet Sauvignon by “AR 2000”, we can conclude that second group of wines treated with “AR 2000” had a higher abundance of alcohols. Apart from 3-methylbutanol, the major alcohols in wine samples were 2-methylpropanol, hexanol and phenylethyl alcohol. Their quantities were in good correlation with the concentration of alcohols in free and glycosidically bound forms in Muscat “á petit grains” wines, in red wine from “*Vitis vinifera* cv. Castañal” grown in Galicia, “Muscadine grape juices” and red wines of “*Vitis Vinifera* L. cv. Őkűzgőzű and Bogazkere” grown in Turkey (Sánchez Palomo *et al*., 2006; Vilanova *et al*., 2007; Baek *et al*., 1997; Cabaroglu *et al*., 2002).

Although most of the alcohols are potent odorants, they can have a negative effect on wine flavour if their concentration exceeded 400 mg/L (Gómez García-Carpintero *et al*., 2011). Hexanol found in relatively higher concentration in free form in some red and white wines shown in Table 11 might have negative effect on the overall flavor of wines with typical unpleasant “vegetal” or “herbaceous” flavor (Gómez García-Carpintero *et al*., 2011). The higher level of benzyl alcohol detected only as bound form with maximal concentration of 631.39 μg/L can be explained as a product of metabolic activity of yeasts or as wine oxidation product of benzaldehyde (Genovese *et al*., 2007).

3.4.3 Volatile phenols

Volatile phenols are formed via decarboxylation of hydroxycinnamic acids being naturally present in grapes. More precisely, the most dominant volatile phenol 4-vinylguaiacol is a derivative of coumaric and ferulic acid. Its maximal concentration of 4.14 mg/L found in “Muscat Ottonel” wine

was in good agreement with concentration of the same compound quantified in Muscat grapes (Kang *et al*., 2010).

3.4.4. Aldehydes

The most abundant aldehyde in wines was furfural quantified in a concentration of 1.33 mg/L. It is formed as a product of degradation of carbohydrates in wines during aging (Genovese *et al*., 2007).

Benzaldehyde is a very potent aldehyde and its smell is described as "almond-like". This aldehyde was only found in a bound form in wines with concentrations from 21.89 μg/L to 47.24 μg/L. Acetaldehyde is formed by enzymatic oxidation of benzyl alcohol and its higher quantities in some wines is related to infection of grapes with *Botrytis cinerea*. The quantities of liberate bound form of benzaldehyde was the same in wines from Muscat "á petits grains" grapes in the work of Castro Vázquez *et al*. (2002).

3.4.5. Esters and acetates

Two types of esters are present in wines: the acetate of higher alcohols which are formed by reaction of acetyl CoA and higher alcohols and the esters of fatty acids and ethanol which are synthesized from acyl CoA and alcohols. Esters occur in all studied samples as the major volatile constituents. Their odor description in the published literature is described as "fruity" (Gómez García-Carpintero *et al*., 2011).

Ethyl acetate was quantified in Macedonian wines in concentrations of 28.45 mg/L. This concentration did not exceed the critical abundance when it can generate a "sour vinegar" odor. Other esters such as hexyl acetate and benzyl acetate have powerful fruity flavors and they are produced during

cold maceration at temperature below 15 °C. Other esters such as ethyl hexanoate, ethyl octanoate and ethyl decanoate are responsible for the flavor of red wines. Usually their concentrations are markers for the quality of red wines. However, some of the esters present in normal concentration in wines such as ethyl lactate and diethyl succinate do not have significant contribution to the overall flavor of the wines. The detected high concentrations of esters in all analyzed samples are in good agreement with the quantities of esters in wines from "Chardonnay" and "Pinot Gris" extracted by Divinylbenzene-Carboxen-Polydimethylsiloxane (DVB/Carboxen/PDMS) fiber and HS-SPME in the study of Howard *et al*. (2005). Their concentrations are independent from the activity of "AR 2000" (Table 13).

3.4.6. Acids

Acetic acid can be produced from acetic bacteria or as a product of ethanol oxidation during alcoholic and malolactic fermentation. Its concentration is an indicator of lactic and acetic bacterial activity in the wines. In higher concentration acetic acid gives rise to an undesirable aroma and the taste. The wine can be very "sour" with an unpleasant "vinegar" scent.

Fatty acids are products from yeast activity during winemaking and their production is governed by the initial composition of the must and fermentation conditions (Gómez García-Carpintero *et al*., 2011). High concentrations of hexanoic, octanoic and decanoic acid in analyzed wines can be products of lipid oxidation. The concentration of fatty acids found in white and red wines from Macedonia were in good agreement with their concentrations estimated by using two extraction procedures HS-SPME and XAD-2 resin in the work of Bohlscheid *et al*. (2006).

3.4.7. Effects of treatment with "Endozym Aromatic" and "AR 2000" enzymes on wine volatiles

The enzyme "Endozym Aromatic" is known to act as pectolytic enzyme for better extraction of free volatile compounds as well as β-glycosidase allowing the glycosidic cleavage of bound volatile compounds.

White wines (V1-V9) produced from "Muscat de Frontignan" variety of grapes were produced with "Endozym Aromatic" (as it is described in the section of winemaking procedure). If we compare the results from analyses of wines V1-V9 presented in Table 12 we see that terpenes have a significant impact on the flavor of Temjanika and Muscat wines. Limonene as the terpene being present in largest amouns in this grape variety might have the highest impact on Temjanika wines due to its "citrus-like" flavor. However, changes in concentrations of volatile compounds, especially terpenes, differ from wine to wine depending on maturity of the grapes, wine-making conditions in different wineries as well as the aging in bottles and conditions of storage.

Pectinase AR 2000 has multiple glycosidase activities. It is known to possess all glycosidase activities required for the release of bound monoterpenyl aglycons (Baek *et al*., 1997).

Wines (V10-V15) were treated with "AR 2000" in order to release the bound volatile compounds. As it is indicative from Table 13, the highest influence of Pectinase "AR 2000" was on the levels of alcohols. The most significant effect of pectinase "AR 2000" was detected on benzyl alcohol (wines V13 and V14). On the other hand, it was noted that benzyldehyde was absent in the same wines. According to the results from the work of Castro Vázquez *et al*. (2002) significant quantities of benzaldehyde were only present in must of grapes before fermentation.

If we compare the volatile profile of wines produced from “Muscat de Frontignan” with the volatile profile of enzymatically treated “Muscat Ottonel” wine a similarity between these two varieties is obvious. The estimation confirmed that during winemaking of wines from “Muscat” variety it is necessary to include enzymatic treatment in order to release the bound volatiles for increasing the overall flavor impression of the wines.

For the analysis of the impact of enzymatic treatment the principal component analyses (Fig. 79 and 80) was performed. The results indicated that the first principal component explained 90 % of the total variability. As it is presented in Fig. 79 the wines are separated in two groups regarding enzymatic treatment. As is shown in Fig. 79, Temjanika wines (V1-V9) produced from “Muscat de Frontignan” grape variety treated with “Endozym Aromatic” and treated wines (V11-V15) with “AR 2000” belong to the same group of classified wines. The second separated group in the PCA plot were wines without enzyme treatment. Only wine V10 produced from Riesling grape variety was classified in the same group with not treated wines since the volatile profile of this wine was not significantly changed after the applied enzymatic treatment. The non-treated wine V12 from Vranec grape variety did not belong to any of the two groups since its volatile profile was significantly different from all of the other wines. As it was presented in Fig. 78 only Vranec wine had the highest level of total alcohols. The other wines presented on the same histogram had esters as the most predominant group of volatiles.

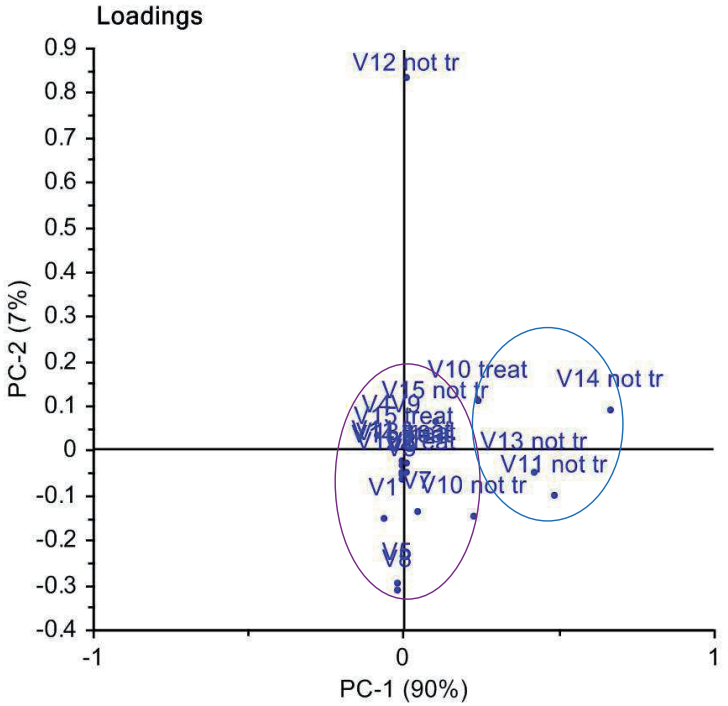


Fig. 79. PCA scater plot of Temjanika, Chardonnay, Vranec, Merlot, Cabernet Sauvignon and Muscat Ottonel wines with and without ezymatic treatment

If we consider the score plot presented in Fig. 80 we can observe the most important components responsible to the classification of the wines in the PCA score plot in Fig. 79. We can conclude that the monoterpene *p*-cymene, alcohols as hexanol and phenylethyl alcohol were mainly responsible for the separation of the wines into two groups on the PCA score in Fig. 79. More precisely, the most significant components for the separation of the wines into two groups were those which concentrations were highly affected by enzymatic treatments. Statistical analyses confirmed that the effect of enzymatic treatment for the examined wines was more significant than the varieties of grapes from which the wines

were produced. Results from this examination confirmed that the type of enzyme was not significant since the wines treated with "Endozym Aromatic" and "AR 2000" were classified in the same group of treated wines.

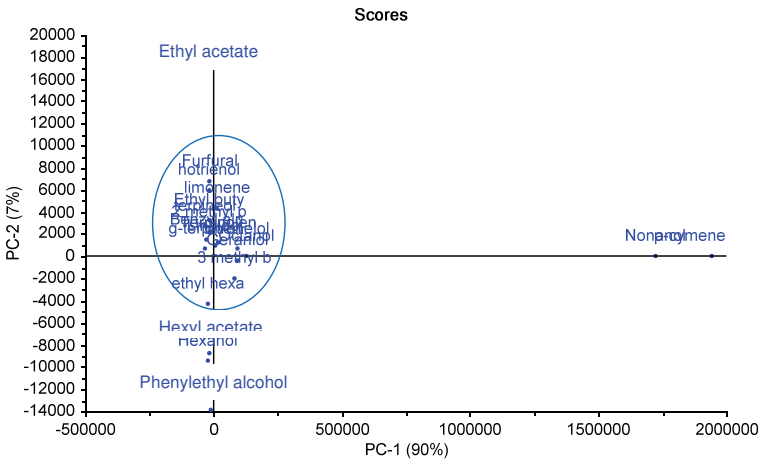


Fig. 80. PCA scater plot of the volatile components from Temjanika, Chardonnay, Vranec, Merlot, Cabernet Sauvignon and Muscat Ottonel wines with and without enzymatic treatment

4. SUMMARY

Results with regard to the stilbene content in the first chapter of this dissertation showed a significant influence of vinification procedures on the concentration of *trans*-resveratrol and *trans*-piceid in Macedonian red wines (Vranec and Merlot grape varieties).

Regarding the variety of grapes, Merlot wines showed higher concentration of *trans*-piceid compared to Vranec wines with concentration range of 0.13-2.24 mg/L. The concentration of *trans*-resveratrol in Merlot wines was in the range of 0.22-1.75 mg/L. No detectable quantity of *trans*-resveratrol was observed in Vranec wines.

The strongest influence on the concentration of stilbenes in wines was due to maceration time. The wines from both varieties showed the lowest concentration of *trans*-resveratrol and *trans*-piceid for 3 days of maceration time and the highest concentration in wines with 6 and 10 days of maceration time.

The lowest abundance of *trans*-piceid was detected in Vranec wine V3 produced with 3 days of maceration time, Macedonian yeast “Vinalco” and 70 ppm of SO_2. The highest concentration of *trans*-piceid and *trans*-resveratrol was detected in Merlot wine M12 with 10 days of maceration time using French yeast “Levuline CHP” and 70 ppm of SO_2.

Results from Trolox Equivalent Antioxidant Capacity (TEAC assay) of Vranec and Merlot wines showed strong relationship between the values of the antioxidant activity and the applied wine-making technology. The results obtained in the second chapter allow the following conclusions:

4.Summary

- There is a strong relationship between antioxidant activity and maceration time. The highest values for antioxidant activity were obtained for 10 days of maceration time in both varieties of red wines.

- A tendency of slightly higher values of antioxidant activity was observed for wines produced by application of Macedonian yeast "Vinalco" compared to French yeast "Levuline CHP".

- The highest values of antioxidant activity were measured for wines from Vranec grape variety with 10 days of maceration time. The longer contact between skin and seeds of grapes during maceration allows a better extraction of polyphenolic compounds.

- Comparison between antioxidant activity of Vranec and Merlot wines under the same wine-making conditions (same type of yeast, equal concentration of SO_2) revealed that Vranec wines have a higher antioxidant activity than Merlot wines.

- Vinification technology had a high impact of sensory analyses of the produced wines. Wines produced by prolonged maceration time of 6 and 10 days and application of Macedonian yeast "Vinalco" had a better taste and astringency due to the higher quantity of extracted polyphenols.

The third chapter of this PhD dissertation described the isolation of high valuable natural pigments (anthocyanins-3-glucosides) from grape pomace. Countercurrent chromatography was applied for the isolation of the pigments and high performance liquid chromatography, mass spectrometry and NMR spectroscopy for structure confirmation of isolated anthocyanins-

3-*O*-glucosides. Quantities of isolated pigments indicated that the pomace from "Vranec" grape variety was the richest source for malvidin-3-*O*-glucoside and malvidin-3-*O*-*p*-coumaroylglucoside. The experiments showed that from 500 g of grape pomace obtained after 20 days of maceration time 19 mg of pure malvidin-3-O-glucoside and 5.5 mg of malvidin-3-*O*-*p*-coumaroyl-glucoside can be isolated. Delphinidin-3-*O*-glucoside, cyanidin-3-*O*-glucoside, petunidin-3-*O*-glucoside and peonidin-3-*O*-glucoside were isolated in lower quantities.

The "color activity concept" explains the contribution of the most abundant anthocyanins-3-*O*-glucosides to the color of the three varieties of grape pomaces (Vranec, Merlot and Pinot Noir). Petunidin-3-*O*-glucoside contributed more significant to "Pinot Noir" grape pomace in comparison with the other two varieties. The violet color of "Vranec" grape variety was attributed to the significant quantity of malvidin-3-glucoside and the blue shadow was attributed to malvidin-3-*p*-coumaroylglucoside.

In the last chapter of this dissertation, the volatile profiles of Macedonian white and red wines were studied by using head space solid phase microextraction (HS-SPME) and gas chromatography mass spectrometry (GC-MS). Terpenes, alcohols, esters and fatty acids were identified as the most important volatiles which contribute to the overall flavor of the wines. White wines produced from Muscat varieties "Muscat de Frontignan" and "Muscat Ottonel" had significant quantities of limonene, linalool, geraniol, nerol, β-citronellol, α-terpineol and α-terpinolene since the flavor of those wines are strong dependent to the concentration of monoterpenes. On the other hand, wines produced from Riesling, Chardonnay, Merlot, Vranec and Cabernet Sauvignon contained alcohols and esters as the most abundant group of volatile compounds.

4.Summary

The effect of the enzymes “Endozym Aromatic” and “AR 2000” on the volatile profile of wines was studied. Enzyme treatment with “AR 2000” had the most significant effect on alcohols and fatty acids in wines from Riesling, Chardonnay, Merlot, Vranec and Cabernet Sauvignon. The volatile profile of enzymatically treated wine from “Muscat Ottonel” grapes was very similar to the treated wines from “Muscat de Frontignan” since the terpenes were one of the major volatiles responsible for the smell and the taste of the wine. Liberated geraniol and nerol in concentrations of 1526.4 μg/L and 1688.2 μg/L as well as nerol in concentration of 350.12 μg/L indicated that enzymatic treatment is necessary for improving the volatile profile of Muscat wines.

The results from principal component analyses (PCA) showed that type of enzyme was not significant since the wines treated with “Endozym Aromatic” and “AR 2000” were classified in the same group of treated wines.

5. MATERIALS AND METHODS

5.1. Wines and by-products

5.1.1. Wines produced under different wine-making technology

The content of *trans*-piceid and *trans*-resveratrol were determined in red wines from grape varieties Vranec and Merlot from 2007 vintage year. Also, their antioxidant activity was object of study.

Wine-making procedure of Vranec and Merlot wines

Twelve red wines from Vranec grape variety (V1-V12) and twelve red wines from Merlot variety (M1-M12) produced at the Experimental Laboratory of the Department for Enology, Institute of Agriculture, Skopje, Macedonia, were subject of investigation (Table 14).

Grapes from both varieties were harvested at optimal maturity (22 ºBrix for Vranec and 20 ºBrix for Merlot) and after crushing the grape mash was divided into 12 lots collected in 25 L plastic fermentation tanks. Aqueous solutions of potassium metabisulfite was added to the mashes of both varieties and mixed to give six tanks with 30 mg/L total SO_2 and six tanks with 70 mg/L total SO_2. Two yeast strains (*Saccharomyces cerevisiae*) were used for fermentation: Vinalco, selected by Yeast Factory, Bitola, R. Macedonia, and Levuline, isolated in the terroirs of Champagne and selected by CIVC 8130 (Interprofessional Committee of Champagne Wines), France. Vinalco (20 g/100 L) was applied to three lots containing 30 mg/L SO_2 and three other lots containing 70 mg/L SO_2 of each variety Levuline (30 g/100 L) was applied to the other lots of both varieties containing either 30 mg/L SO_2 or 70 mg/L SO_2.

Table 14. Labels of Vranec (V1-V12) and Merlot (M1-M12) wine samples prepared under different vinification procedures

days of maceration		*Type of yeast*	*Conc. of SO_2 (mg/L)*	*days of maceration*		*Type of yeast*	*Conc. of SO_2 (mg/L)*
V1	3	Macedonian	30	M1	3	Macedonian	30
V2	3	French	30	M2	3	French	30
V3	3	Macedonian	70	M3	3	Macedonian	70
V4	3	French	70	M4	3	French	70
V5	6	Macedonian	30	M5	6	Macedonian	30
V6	6	French	30	M6	6	French	30
V7	6	Macedonian	70	M7	6	Macedonian	70
V8	6	French	70	M8	6	French	70
V9	10	Macedonian	30	M9	10	Macedonian	30
V10	10	French	30	M10	10	French	30
V11	10	Macedonian	70	M11	10	Macedonian	70
V12	10	French	70	M12	10	French	70

5.1.2. Commercial wines

Volatile profile of 15 of the most popular white and red wines from Macedonia was object of study in this PhD work. The labels of wines as well as the wineries from which samples were obtained are listed in Table 15.

Wine-making procedure of Temjanika wines

Grape from Muscat de Frontignan variety (known in Macedonia as Temjanika) was harvested at optimal maturity. After crushing the grape mash, aqueous solutions of potassium metabisulfite was added with 50

mg/L total SO_2. The must was obtained by pressing using vacuum press and liquid CO_2 for protection of mush from oxidation. Extraction of polyphenolic compounds was obtained by 4 h maceration time of the skin and seed from the grape. Futhermore, liquid enzyme "Endozym Aromatic" for aromatic extraction (AEB, Bresica, Italy) was added in a dosage of 2g/hL to the must. The double role of this enzyme is due to better extraction of free aromatic compounds by its pectolytic activity and acting on aroma precursors by its β-glycosidasic activity. Sedimentation of the must was obtained during 48 h at 8-10 ºC. Two yeasts, "Fermol Arôme Plus" and "Fermol Charmat" (*Saccharomyces cerevisiae*) obtained from the same Italian company were used in dosage of 20 mg/100 kg grapes. The selection of yeasts was due to their ability to generate aroma precursors and to produce esters and acetates during fermentation at low temperature. Cold maceration was applied at 12-14 ºC during alcoholic fermentation. Aging of the wines in bottles was for 2 years.

Wine-making procedure of Muscat Ottonel wine

Grapes from Muscat Ottonel variety were harvested at optimal maturity (pH 3.4, 230g/L sugar and total acidity of 4.9 g/L). After crushing and addition of dry ice and nitrogen for protection of oxidation, the cold maceration was performed for 9 h at 10 ºC. After cold maceration the grape mash was pressed. The obtained grape juice with turbidity of 85 nephelometric turbidity units (NTU) was inoculated with *S. cerevisiae* ph.r. *cerevisiae* yeast in concentrations of 25 g/hL. Alcoholic fermentation during 2 weeks was performed at a temperature between 12-15 ºC and produced a young wine with 1g/L of residual sugar. After

filtration the cold stabilization of the wine was performed at –6 ºC. During cold stabilization PVPP agent was added for removing polyphenols extracted from seed and skins of the grape. The wine was filtrated (first filtration with 60 μm and second with 40 μm filter) and bottled. Aging of the wines in the bottles was during 1 year.

Table 15. Labels of commercial white and red wines

		Name	***Variety***	***Vintage***	***Winery***
1.	White wine	Temjanika	Muscat de Frontignan	2008	Tikveš
2.	White wine	Temjanika	Muscat de Frontignan	2007	Tikveš
3.	White wine	Temjanika	Muscat de Frontignan	2006	Tikveš
4.	White wine	Temjanika	Muscat de Frontignan	2008	Popov
5.	White wine	Temjanika	Muscat de Frontignan	2008	Popova Kula
6.	White wine	Muscat	Muscat de Frontignan	2007	Bovin
7.	White wine	Muscat	Muscat de Frontignan	2006	Bovin
8.	White wine	Muscat	Muscat de Frontignan	2007	Skovin
9.	White wine	Muscat	Muscat de Frontignan	2006	Skovin
10.	White wine	Alexandira Riesling	Riesling	2008	Tikveš

Table 15. Labels of commercial white and red wines (Cont'd)

		Name	***Variety***	***Vintage***	***Winery***
11.	White wine	Bistro Bianco	Chardonnay	2008	Fonko
12.	Red wine	T' ga za Jug	Vranec	2008	Tikveš
13.	Red wine	Makedonsko Crveno	Merlot	2008	Skovin
14.	Red wine	Alexandra Cabernet Sauvignon	Cabernet Sauvignon	2008	Tikveš
15.	White wine	Muscat Ottonel	Muscat Ottonel	2009	Stobi

5.1.3. Grape pomace samples

The winery Elenov from Macedonia kindly provided samples of three varieties of red grape pomace: "Vranec", "Merlot", and "Pinot Noir". The monovarietal samples of grape pomace were obtained from the 2009 vintage. Wine-making included 20 days of maceration time. From each variety, 1.5 kg of grape pomace was washed with Nanopure® water for removing polar impurities and sugar. To remove water, the samples were lyophilized. After lyophilization, the dry grape pomace was crushed in powder. 500 g of powdered grape pomace from each variety was used for extraction of anthocyanins.

5.2. Chemical and standards

Trans-resveratrol and *trans*-piceid *(trans*-resveratrol-3-glucoside) were obtained from Phytolab, Germany. Distilled water, acetonitrile, methanol and glacial acetic acid were purchased from Merck, Germany. 2,2-Azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS•+) in the crystallized diammonium salt form, horseradish peroxidase type VI-A, hydrogen peroxide 30 % (v/v), Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid, a water soluble tocopherol analogue) were from Merck, Germany. 2-nonanal used for quantification was purchased from Merck, Germany. SPME cartridge 65µm PDMS/DVB coating fiber, 50/30 µm CRB-DVB-PDMS fiber, 85 µm Carboxen/PDMS and 85 µm polyacrylate coating fiber was purchased from Sigma Aldrich, Belgium.

5.3. Extraction of anthocyanins from grape pomace

The samples were defatted with (3 x 200mL) of hexane. For extraction of anthocyanins mixture of methanol/formic acid (19:1 v/v) was used. For complete extraction the suspension was stored at room temperature overnight.

5.3.1. Solid phase extraction and purification of crude extract of anthocyanins from grape pomace

Solid phase extraction (SPE) is a separation process by which compounds which are dissolved or suspended in a liquid mixture are separated from the other compounds in a mixture according to their physical and chemical properties.

Extracts obtained from all varieties of grape pomace after evaporation of solvents were viscous. To remove sugars, organic acids, proteins, and salts XAD-7 column (45 x 5 cm) was used. The columns were washed with Nanopure® water and after that the anthocyanins were eluted with a mixture of methanol/formic acid (19:1 v/v). After evaporation the samples were freeze dried to give the anthocyanin enriched XAD-7 extract.

5.4. HPLC analysis

5.4.1. HPLC-ESI-MS and HPLC-DAD analysis for resveratrol determination in Vranec and Merlot wines

A Chromatograph Agilent Technologies 1200 Series, with Jasco AS-950 Sampler, an auto injector (20 µL injection volume) was used for the analyses. Mass selective detector type Bruker Daltonics HCT Ultra was used for identification of *trans*-resveratrol (under negative ion mode) and *trans*-piceid. For quantification purposes, a Jasco MD-1510 Multiwavelength Detector was applied. Separation of the components was performed by using a C18 Luna column (5µm x 4.6 mm x 25 cm). The mobile phase flow rate was 0.5 mL/min. The eluents were: solvent A: water/acetic acid (98:2, v/v) and solvent B: 100 % acetonitrile, with gradient elution: 0-5 min, 85-80 %; 5-15 min, 80-72 %; 15-50 min, 72-58% and 50-52 min, 58-0 % solvent A. Quantification of *trans*-resevaratol and *trans*-piceid was performed with external calibration using a standard solution of resveratrol and its glucoside at a wavelength of 306 nm. *Cis*-configured forms of resveratrol and piceid at 286 nm were below limit of quantification in all wines and below limit of detection in the most of the wines.

Standard solutions used for the calibration curve were prepared in methanol and kept in the dark at –5 ºC. The calibration curves y=12323x-1779.7 for *trans*-resveratrol and y=17008x-11211 for *trans*-piceid are shown in Fig. 81 and 82. They were established by plotting the peak areas against different concentrations of *trans*-resveratrol (varying from 0.1 to 20 mg/L) and for *trans*-piceid (varying from 0.5 to 21 mg/L) with correlation factors of 0.9998 for *trans*-resveratrol and 0.9992 for *trans*-piceid, respectively.

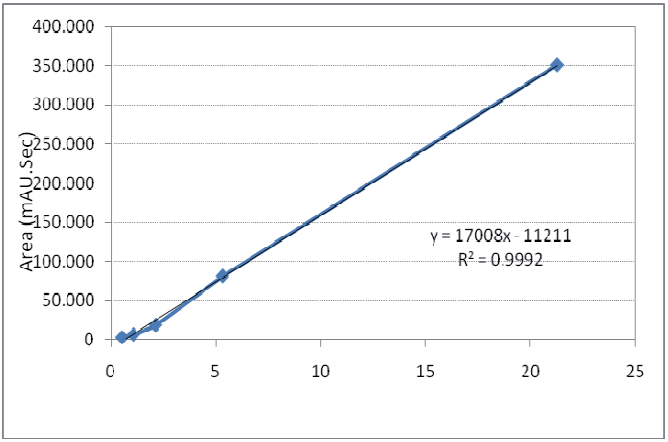


Fig. 81. Calibration curve of *trans*-piceid (mg/L)

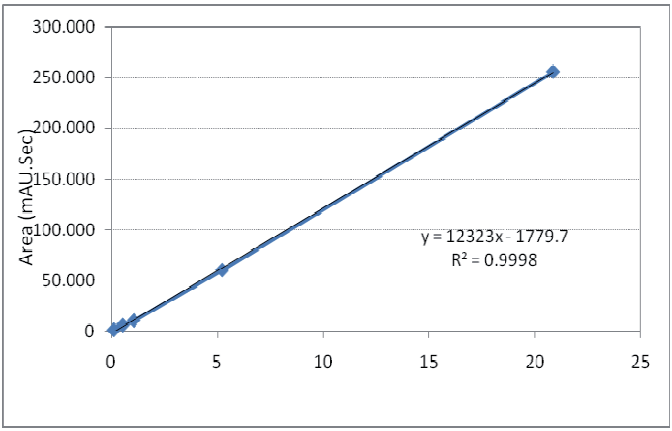


Fig. 82. Calibration curve of *trans*-resveratrol (mg/L)

5.4.2. HPLC-ESI-MS and HPLC-DAD for determination of anthocyanins from grape pomace

Separations of CCC fractions were carried out on a Luna 5µ C18 column (250 x 4.6 mm) with guard column. A binary gradient of a mixture of water/acetonitrile/formic acid was used. Linear gradient was as follows: A 83/7/10; B: 40/50/10 (v/v/v); % A: 0 min 94 %, 20 min 80 %, 35 min 60 %, 35 min 40 %, 40 min 90 %, 45 min and 55 min 94 %. The flow rate was set at 0.5 mL/min. Detection was either done by DAD (Jasco MD-1510) or ESI-MS2 (Bruker, Esquire) in the positive ion mode. Data about the purity of anthocyanin fractions is based on the DAD chromatogram using $\lambda = 520$ nm as detection wavelength.

5.4.3. Preparative HPLC

Purification of anthocyanins fractions was made by using preparative HPLC with a Knauer (Berlin, Germany) HPLC pump 64 and UV/VIS detector. The purification of CCC fractions was carried out on Luna RP-18

column (Phenomenex, Germany) at a flow rate of 0.5 mL/min. The two solvent systems A and B were used. Solvent A was mixture of water/acetonitrile/formic acid 87:3:10 (v/v/v), and solvent B was mixture of water/acetonitrile/formic acid 40:50:10 (v/v/v). The linear gradient was as follows: 0 min, 6 % B; 20 min, 20 % B; 35 min, 40 % B; 40 min, 60 % B; 45 min, 90 % B; 55 min, 6 % B. The obtained chromatograms were interpreted by using Borwin PDA chromatography software (Jasco, Groß Umstadt, Germany) and the purified peaks from anthocyanins were detected using λ = 520 nm as detection wavelength.

5.5. Spectrophotometric analysis for determination of antioxidant activity of wines

UV/VIS spectrophotometer Bruker IFS 66 was used for the analyses. The absorbance was measured at 734 nm at room temperature.

The antioxidant activity of wines was determined using the $ABTS^{\bullet+}$ method for screening of the antioxidant activity as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates and carotenoids (Rice-Evans *et al*., 1996).

The assay involves direct production of the blue/green $ABTS^{\bullet+}$ chromophore through reaction between $ABTS^{\bullet+}$ and potassium persulfate. The product has an absorption maxima at 414, 645, 734 and 815 nm (Villaño *et al*., 2004).

The method employed in this study gives a measure of the antioxidant capacity of red wines produced under different conditions.

For this purpose 10 mL of ABTS solution was prepared. The ABTS solution was made from 38.43 mg of ABTS and 6.90 mg of $K_2S_2O_2$, and made up with Nanopure® water to volume.

For the calibration curve (shown in Fig. 83) 5 mL solution of 12.52 mg of Trolox standard were diluted with ethanol (97 %) and four standard solutions were prepared for calibration curve with concentration of 250, 500, 750 and 1000 mmol/5mL, respectively. The measured values for antioxidant activity of the wines were recorded after 6 min.

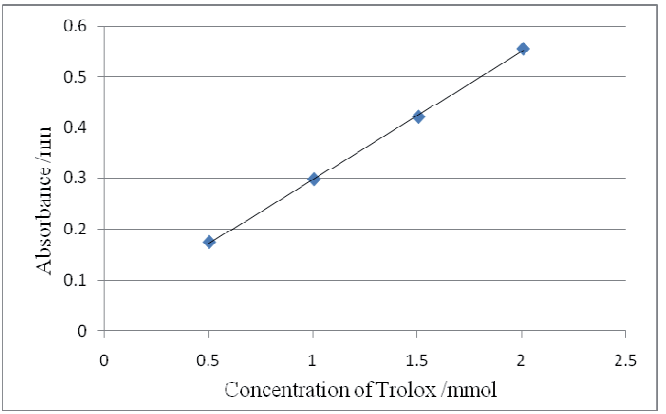


Fig. 83. Calibration curve of Trolox solution

5.6. Countercurrent chromatography

Countercurrent (CCC) liquid–liquid chromatography is a method for separation and isolation of compounds from complex mixtures without solid stationary phase. This separation technique established in the early 1970s has advantage over the conventional column chromatography by eliminating use of a solid support where the amount of stationary phase is limited and irreversible adsorption from the support occurs (Winterhalter, 2007). Separation is achieved by a two-phase solvent system.

High speed countercurrent chromatography (HSCCC) which was developed in 1980s improved partition efficiency including better resolution and shorter separation times (Degenhardt *et al*., 2000b). In

HSCCC the solvents are filled in a teflon tube which is wound around a coil. These coils are usually connected in series. A planetary rotation of the coils creates an alternating force field in the tube and mixing and settling zones are generated. Thus, the sample is continuously distributed between the two phases during separation.

For successful isolation of anthocyanins, the selection of suitable solvent mixtures was performed, for their evenly distribution between the two phases. This can be observed visually by mixing an aliquot of the sample with the two phases in a test tube.

A CCC-1000 high-speed countercurrent chromatograph (triple coil, ID 2.6 mm, total volume 850 mL, 800 rpm, Pharma-Tech, USA) was used (Fig. 84). MTBE/n-butanol/ acetonitrile/water 2:2:1:5 v/v/v/v acidified with 0.1 % trifluoracetic acid was used as solvent system. The lower layer was the mobile phase and elution mode was head to tail. Flow rate was set at 3 mL/min. Detection was carried out at λ = 520 nm (Knauer Variable Wavelength Monitor). 1.0 g of the respective freeze-dried XAD-7 extract from each variety of grape pomace was separated in each run.

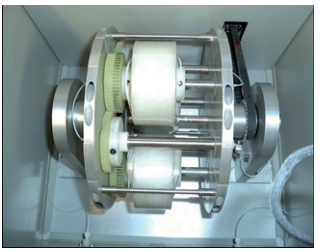


Fig. 84. Countercurrent chromatograph

5.7. NMR spectroscopy

Nuclear Magnetic Resonance Spectroscopy (NMR) was applied for structure elucidation of isolated anthocyanins.

^{1}H and ^{13}C NMR spectra were measured on a Bruker AMX 300 spectrometer (Bruker Biospin, Germany) at 300.13 and 75.49 MHz, respectively. Anthocyanins were dissolved in a mixture of methanol-*d*4/TFA-*d*1 (19:1, v/v). Data were processed by WIN NMR software version 6.1.0.0.

5.8. "Color activity concept"

The "color activity concept" was applied in order to find the impact of isolated pigments on the color of grape pomace. The calculation of color activity values is based on the ratio of concentration of the pigment and its visual detection threshold. For that purpose pure anthocyanins were dissolved in buffer solution with pH 3.0 and two solutions A and B were prepared.

The solution A was prepared by dissolving of 21 g of $C_6H_8O_7$ x H_2O per liter Nanopure® water. The solution B was prepared by dissolving of 36, 5 g of $NaHPO_4$ x $2H_2O$ per liter water with Nanopure® grade. The buffer solution with pH 3 was prepared by mixing of 79.45 mL of solvent A and made up to 100 mL with solvent B.

Most natural pigments are used for coloring food and beverages. The usual pH value of soft drinks is 3.0. That was the reason of testing the color activity index in buffer solution of pH 3.0.

5.9. Optimization of the head-space solid phase microextraction (HS-SPME) procedure for determination of volatile profile of commercial wines

Solid Phase Microextraction (SPME) developed in the early 1990s is a simple and inexpensive preparation technique where no solventsare required. SPME involves the use of a fiber coated with an extracting phase that can be a liquid (polymer) or a solid (sorbent), which extracts different kinds of analytes (including both volatile and non-volatile) from different kinds of media that can be in liquid or gas phase (Tat *et al*., 2005). SPME technique can be used for head space analysis or direct extraction of the analytes from liquids (Fig. 85).

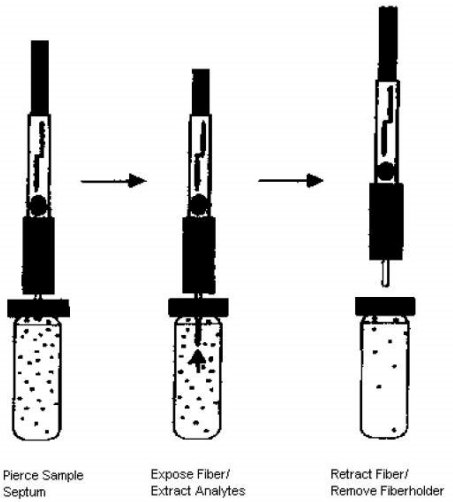


Fig. 85. Solid Phase Microextraction

For optimization of the method for isolation of volatile compounds, red and white model wine was used. The following parameters were optimized:

fiber type, contact time between fiber and headspace, sample temperature and addition of salt.

Type of fiber

Extraction of volatile compounds from white and red wines with different types of fibers was performed. For this purpose SPME cartridge 65µm PDMS/DVB coating fiber, 50/30 µm CRB-DVB-PDMS fiber, 85 µm Carboxen/PDMS and 85 µm polyacrylate coating fiber were used.

Absorption time between fiber and head-space

Different contact times of the fibers to the sample head-space (HS) 20, 30 and 40 min. were evaluated. 20 min of extraction time was not sufficient for complete extraction of polar compounds even by application of 85 µm polyacrylate fiber. The peak area of some high volatile compounds was lower during 40 min. compared to 30 min. contact time which indicated that desorption of high volatile compounds occurred. Furthermore, in all analyses, optimal time for extraction of volatile and semivolatile components in headspace mode of 30 min. was used.

Temperature

The effect of temperature on the extraction of volatile compounds from wine was studied in white and red wine samples at 20 ºC, 30 ºC, and 50 ºC. The best extraction temperature for red and white wines was 30 ºC. All analyses were done in duplicate.

Salt addition

Effect of salt addition in wines was different according to different types of fibers. Extraction of volatile compounds by using 50/30 μm CRB-DVB-PDMS and 65μm PDMS/DVB coating fiber was higher with addition of 0.5 g of NaCl in order to increase the concentration of the volatile compounds in headspace. In the case of 65μm PDMS/DVB and 85μm polyacrylate fiber no higher areas of peaks after addition of salt could be detected.

5.10. Isolation of free volatile compounds from wines

Isolation of wine volatiles was performed with a 50/30 μm CRB-DVB-PDMS (Carbowax-Divinylbenzene-Polydimethylsiloxan) fiber. Optimal conditions for wine sample preparation were the following: 30 min continuous stirring of 2 mL of wine in 5 mL vial at 30 °C and addition of 0.5 g of NaCl. Before each exposure, the fiber was cleaned in the injection port at 260 °C for 5 min. All analyses were performed in duplicate.

5.11. Isolation of bound volatile compounds from wines by using solid phase extraction and enzyme treatment with "AR 2000"

Isolation of bound volatile compounds from wines was performed by using a glass column packed (45x5 cm) with XAD-2 resin. The volatile components of 150 mL of each wine was eluted with 500 mL of Nanopure® water and 500 mL of solution pentane/diethyl ether (1:1) to remove all volatile components. After that the bound volatile extract of wine was eluted with 500 mL of methanol. After the evaporation (40 ºC) the extracts

were dried by lyophilization. For incubation of "AR 2000" enzyme, the buffer of citric acid and dinatriumhydrogenphosphate dihydrate was prepared. Solvent A was prepared by dilution of 1.921 g of anhydrous citric acid with Nanopure® water till 100 mL. Solvent B was prepared by dilution of 3.560 g of dinatriumhydrogenphosphate dihydrate with Nanopure® water till 100 mL. By mixing of 48.5 mL of solvent A and 51.5 mL of solvent B a buffer with pH 5.0 was obtained.

Wine extracts of each wine obtained using solid phase extraction was diluted with 10 mL of buffer solution. To each wine extract was added 3 mg of "AR 2000" enzyme and incubation was performed at 40 ºC during 18h.

2 mL of wine extract obtained by enzymatic breakage of bound compounds was inserted into SPME vial and 50 µL of internal standard solution (prepared by dilution of 50 µL of 2-hepatnol in 100 ml of diethyl ether) was added. The extraction of volatile compounds obtained by enzymatic breakage was performed at 30 ºC by 50/30 µm CRB-DVB-PDMS fiber using headspace with addition of 0.5 mg NaCl and 30 min. extraction time.

5.12. Qualitative analysis of volatile compounds

Qualitative analysis of volatile compounds was performed by using Gas Chromatography- Mass Spectrometry (GC-MS) HP 6890 with HP5973 mass selective detector. For identification purposes, NIST and Wiley mass spectra databases as well as a homemade database were used. Separation of the compounds was performed using an HP Carbowax column (60 m x 0.25 mm x 0.25 µm) and identification is based on comparison of retention

time and mass spectra with those obtained from the libraries. The temperature gradient started from 35 °C to 180 °C with step of 3 °C/min and from 180 °C to 260 °C with step of 20 °C/min. Injector temperature was set to 260 ºC; injection mode split with split ratio 1:5; Helium was used as carrier gas, using 35 kPa (32 cm/s); interface temperature was 280 ºC; acquisition mass range, 40-400 m/z; solvent cut, 2 min.

5.13. Quantitative analysis of volatile compounds

For quantification of volatile compounds 2-heptanol was used as internal standard in concentration of 4 μg/L. For this purpose 50μL of 2-heptanol was diluted in 100 mL of diethyl ether. 50 μL of this solution of internal standard was added into SPME vials which contained 2 mL of wine. All analyses were performed in duplicate and the results were expressed as mg of compound per litre of wine. The same temperature gradient used for MS identification was applied for quantification of volatile components in the wines.

5.14. Sensory evaluation of commercial wines

The evaluation of the general organoleptic taste of the wines was obtained by 7 panellists. One panellist was from Department of Enology and six panellists were with previous experience for wine tasting. The panellists were selected on the basis of interest and availability. They were asked to give global sensory quality according to their perception of the taste of the wines. The value ranging was from 1 to 10 for the best taste of the wine using 9 descriptors: sweety, bitter, sour, spicy, raisin, woody, floral, citrus

and artificial note. Randomized samples of wines from 20 mL were served in dark glasses covered with Petri dishes. Water was provided for rinsing the palate during testing. The temperature in the degustation room was 20 °C.

5.15. Statistical analysis

The data obtained for stilbene content and Trolox equivalent antioxidant capacity (TEAC) of Vranec and Merlot wines obtained under different wine-making procedure were subjected to the General Linear Model (GLM). The applied statistical treatment allowed to determine the significant main and interaction effects ($p < 0.05$) of independent variables namely, type of yeast (Macedonian yeast, Vinalco and French yeast, Levuline), maceration time (3, 6 and 10 days) and SO_2 content (30 and 70 mg/L) on the level of *trans*- resveratrol and *trans*- piceid and antioxidant activity of 24 Macedonian red wines from both varieties, Vranec and Merlot. The corresponding variables are statistically significant ($p < 0.05$) if the absolute F ratio becomes larger and the *p*-value becomes smaller. The data analysis was performed by using the Minitab software v. 13.2 (Minitab Inc., State College, PA, USA).

The data obtained for volatile and sensory analysis of wines were subjected to Cluster and Principle Component Analysis. Data analysis obtained for TEAC value, volatile and sensory analysis of wines were performed using the Minitab software v. 13.2 (Minitab Inc., State College, PA, USA) and the Unscrambler X10.1. (ADS Inc., USA).

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